EXPERIMENTAL AUTOIMMUNE THYROIDITIS INDUCED IN MICE BY DEFINED NON-DOMINANT THYROGLOBULIN T-CELL EPITOPES

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EXPERIMENTAL AUTOIMMUNE THYROIDITIS INDUCED IN MICE BY DEFINED NON-DOMINANT THYROGLOBULIN T-CELL EPITOPES.

by

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A thesis submitted to the

School of Graduate Studies

in partial fulfilment of the

requirements for the degree of

Doctor of Philosophy in Medicine

Faculty of Medicine
Memorial University of Newfoundland

February 1995

St. John's Newfoundland

Dedicated to my parents,
Vassilis & Maria,
my brothers
Evripithis & Nickos
my sister in law, Katerina
and my niece, Eleftheria

SUMMARY

Experimental autoimmune thyroiditis (EAT) in mice induced by thyroglobulin (Tg) and adjuvant has been studied as a model for Hashimoto's thyroiditis. The disease is MHC-controlled and T-cell mediated but little is known about the nature and the number of immunopathogenic Tg T-cell epitopes. In this study, we attempted to define such epitopes by testing Tg sequences, previously identified as potential T-cell epitopes through the AMPHI and "tetramer motif" algorithms, in various strains of mice for both immunogenicity and pathogenicity. From the three synthetic Tg peptides tested two sequences, TgP1 and TgP2, were found to be pathogenic in classical high responder (H-2^k and/or H-2^s) mice. The third sequence, TgP3, was not pathogenic in mice of k,b,d,s haplotypes. All three sequences were immunogenic in mice because they induced peptide-specific antibodies and/or T cells which were strain dependent. TgP1 and TgP2 were shown to encompass non-dominant determinant(s) at both B- and T-cell levels. Similarly, TgP3 was found to involve non-dominant B-cell epitope(s) although its ability to be recognized by T cells was never tested. EAT induction with defined Tg T-cell epitopes constitutes a system where the fine mechanisms leading to thyroid autoimmunity can be extensively studied at both cellular and molecular levels. In an approach to study these mechanisms using defined Tg peptides, we attempted to map the H-2 region(s) responsible for EAT induction by TgP1. As in Tg-induced EAT, TgP1-induced EAT was shown to be under the direct control of MHC-region products and to follow a pattern similar to Tg disease susceptibility. However, within the k haplotype, expression of H-2E but not H-2A molecules was necessary for EAT induction. Moreover, TgP1 was shown to elicit IgG specific antibodies which were reactive to purified Tg *in vitro* and Tg stored in the lumen of normal mouse thyroids. This finding may imply involvement of TgP1-specific IgG in EAT pathogenicity although such

involvement has not been further investigated in this study. In summary, the application of algorithms for prediction of Tg T cell-reactive sites was proved to be successful and reliable. Defined immunopathogenic Tg T-cell epitopes can be used as tools to study immunoregulation in autoimmunity and to design specific immunotherapeutic strategies.

Acknowledgments

I would like to acknowledge all those people that have assisted and morally supported me throughout this work.

Initially, I want to express my sincere thanks to Dr. Theony Valcana, Professor of Physiology, University of Patras, Greece, who strongly advised and encouraged me to pursue graduate studies in Canada. She has been a wonderful example for me both as a scientist and as a human being and has inspired my work.

I am deeply indebted to Dr. Verna Skanes, Assistant Dean of Research and Graduate Studies and member of my supervisory committee, for the willingness and the time that she so abundantly gave to help me in many ways throughout my studies. Without her support and encouragement, I would not have been able to overcome some oppositions and complete my program.

Sincere thanks also to Dr. George Carayanniotis, my supervisor, for his assistance at the theoretical and experimental aspects of my project during the first two and a half years of my studies and for any constructive feedback thereafter.

I would like to extend my gratitude to:

Dr. Christopher Sharp, Associate Dean of Research and Graduate Studies for his moral support, understanding and encouragement;

Drs Verna Skanes, Bodil Larsen and Sheila Drover for their support, help and encouragement during the preparation for my comprehensive examination;

Drs Verna Skanes and Bodil Larsen for critical review of this manuscript;

Dr. Zetta Tsaltas for critical comments on Chapters 1, 2 and 9 of this thesis;

Dr. Vernon Richardson for his counsel as a member of my supervisory committee;

Dr. Thomas Michalak for his technical advice in tissue immunofluorescence;

Dr. Andrejs Liepins for assistance in photographic work and

Mr. Ed Evelly for expert assistance with the histological sections.

I have no words to express my appreciation to my parents, my brothers and my sister in-law for all the love, understanding and support that they have offered me in the past. Particularly, I thank my brother Evripithis who despite the distance was always available to uplift me every time I was desperate.

Finally, I am deeply indebted to a special friend, Mary Lyons Hicks, who has been always there for me to share the good and the bad times, my dreams and my fears. She has unconditionally supported and encouraged me all the way through this work.

Last, I thank the Faculty of Medicine, the School of the Graduate Studies and the Medical Research Council of Canada for their financial contributions.

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LIST OF ABBREVIATIONS

a.a:	amino-acid
ADCC:	Antibody-dependent complement-mediated cytotoxicity
anti-Tg:	antithyroglobulin
anti-TPO:	anti-thyroid peroxidase
APC:	antigen-presenting cell
ARTA-1:	autoimmune thyroid disease related antigen
BB/W:	Bio-breeding/Worcester
BSA:	bovine serum albumin
BSS:	balanced salt solution
BTg:	bovine thyroglobulin
BUF:	Buffalo
C':	complement
C5-D:	deficient in the fifth complement component
C6-D:	deficient in the sixth complement component
CFA:	complete Freund's adjuvant
con A:	concanavalin A
CS:	Cornell strain
ddH2O:	double-distilled water
DIT:	diiodotyrosine
dMTg:	denatured thyroglobulin
EAE:	experimental autoimmune encephalomyelitis
EAT:	experimental autoimmune thyroiditis
EAU:	experimental autoimmune uveoretinitis
ELISA:	enzyme-linked immunosorbent assay
FBS:	fetal bovine serum
FITC:	fluorescent isothiocyanyate
GD:	Graves' disease
HEL:	hen-egg lysozyme
HLA:	human leukocyte antigen
HPLC:	high-performance liquid chromatography
HT:	Hashimoto's thyroiditis
[^{3}H]TdR:	^{3}H-thymidine
HTg:	human thyroglobulin
IDDM:	insulin-dependent diabetes mellitus
IFA:	incomplete Freund's adjuvant
IFN-γ:	interferon γ
Ig:	immunoglobulin
IL-2:	interleukin 2
IL-4:	interleukin 4
i.p.:	intraperitoneally
IRBP:	interphotoreceptor retinoid-binding protein
kDa:	kilo-dalton
LNC:	lymph node cells

contd.

LPS:	lipopolysaccharide
MBP:	myelin basic protein
MHC:	major histocompatibility complex
MIT:	monoiodotyrosine
mo:	months
MoAbs:	monoclonal antibodies
mRNA:	messager RNA
MTg:	mouse thyroglobulin
mU:	milli-units
NOD:	non obese diabetic
NP:	influenza A nucleoprotein
NWL:	new white Leghorn
OS:	Obese strain
OVA:	ovalbumin
PBS:	phosphate buffered saline
PBST:	PBS-Tween
pI:	isoelectric point
PPD:	tuberculin purified protein derivative
PTg:	porcine thyroglobulin
PTPO:	porcine thyroid peroxidase
RFLP:	restriction fragment length polymorphism
RTg:	rat thyroglobulin
SAT:	spontaneous autoimmune thyroiditis
s.c.:	subcutaneously
SCID:	severe combined immunodeficiency
SI:	stimulation index
T4:	thyroxine
T3:	triiodothyronine
TCR:	T-cell receptor
TEC:	thyroid epithelial cells
Tg:	thyroglobulin
TgPi :	thyroglobulin peptide i (i=1, 2, 3)
Th:	T-helper
TPO:	thyroid-peroxidase
TRH:	thyrotrophin-releasing hormone
TSH:	thyroid-stimulating hormone
TSH-R:	thyroid-stimulating hormone receptor

CHAPTER 1

INTRODUCTION

1.1 THYROID GLAND AND CLINICAL AUTOIMMUNE THYROID DISEASE

1.1.1 Thyroid gland

1.1.1.1 Localization and morphology

The thyroid gland is the largest and first endocrine gland to appear in the human embryo. It begins its development during the fourth embryonic week, assuming its adult position anterior to the trachea near the base of the neck by the seventh week (J. Hansen, 1990). The adult thyroid derives its name from its shield shape configuration (thyreoeidos: *thyreos*, shield; *eidos*, form), (B. Turlington, 1991). It is a bilobed structure with the two lobes joined together by the isthmus which is a bridge of thyroid tissue that runs anterior to the second and third tracheal rings (J. Hansen, 1990).

1.1.1.2 Physiology

The basic function of the thyroid is the production of two iodinated amino-acid hormones, thyroxin (T4) and triiodothyronine (T3). These are passed into the bloodstream affecting many metabolic processes by virtue of their actions on various cells and tissues (B. Turlington, 1991; R. I. S. Bayliss & W. M. G. Tunbridge, 1991). Abnormalities of thyroid hormone production and release result in thyroid disorders. Thus, excessive or insufficient output of thyroid hormones result in the pathological situation of hyperthyroidism and hypothyroidism respectively (B. Turlington, 1991).

1.1.1.3 Histology and ultrastructure

The thyroid gland is organized into spherical cyst-like structures called follicles (reviewed in R. Ekholm & U. Bjorkman, 1990). The thyroid follicle is the primary unit of the gland both in terms of structure and function because within its highly organized cellular compartment, biosynthesis and secretion of thyroid hormones take place.

A thyroid follicle consists of a lumen surrounded by a single monolayer of epithelial cells (follicular or acinar cells) and is enclosed by a thin basal membrane (reviewed in R. Ekholm & U. Bjorkman, 1990). The lumen is filled with a proteinaceous, viscous solution, the colloid, which is retained at that location because tight junctions (desmosomes) join the edges of follicular cells together, limiting its spread. The colloid contains a mixture of proteins, mainly Tg, a 660 kilo-dalton (kDa) glycoprotein, which is the precursor of thyroid hormone, as well as other lower molecular weight iodoproteins and albumin (L. J. DeGroot *et al.*, 1984a). The shape of the follicular cells varies from cuboidal to columnar, depending on the activation status of the thyroid gland. The apical or luminar surface of the follicular cells is characterized by numerous microvillar projections which are transformed to pseudopods after stimulation, thus increasing considerably contact between follicular cells and the colloid. The basal surface of the acinar cells borders on a capillary and is separated from it by a two layer basement membrane (L. J. DeGroot *et al.*, 1984a).

A prominent characteristic of the follicular cells is their "polarity" (reviewed in R. Ekholm & U. Bjorkman, 1990). This is expressed as differences in both the chemical composition between the apical and basal (facing the extrafollicular space) plasma membrane, and the distribution of organelles within the follicular cells. The presence of tight junctions between the follicular cells contributes to the preservation of differences

in composition between the apical and basal plasma membrane because these tight junctions act as barriers inhibiting free diffusion of proteins and lipids (reviewed in R. Ekholm & U. Bjorkman, 1990). Within the follicular cells, the cellular organelles follow a certain distribution pattern; for example, the nucleus is situated in the basal or the central part of the cell and secretory vesicles in the apical zone. Obviously such a cellular polarity and organization evolved to assist certain functions. Within the follicular cells there are two major processes operating in opposite directions. The first process involves production and transportation of Tg to the follicular lumen and the second endocytosis of iodinated Tg and production of thyroid hormones that are delivered at the basal membrane.

A second less abundant endocrine cell population, consisting of about 1% of the thyroid epithelial cell mass, exists within the thyroid. This population is the parafollicular or C cells (B. Turlington, 1991). C cells have no access to the follicular lumen and are characterized by the production of the peptide hormone calcitonin (C. C. Capen, 1991).

The thyroid gland is highly vascularized containing a network of inter- and intra-follicular capillaries lying close to the follicular basement membranes (C. C. Capen, 1991). A network of lymphatics as well as nerve fibers that are mainly sympathetic and occasionally parasympathetic, are also detected (L. J. DeGroot *et al.*, 1984a).

1.1.1.4 Regulation of thyroid function through the hypothalamo-pituitary axis.

The maintenance of normal thyroid function is highly dependent on the tuned interactions among hypothalamus, pituitary and thyroid gland operating through a hierarchical system of hormones and neurotransmitters (I. R. McDougall, 1992a). The

principal modulator of thyroid hormone synthesis and release is thyroid stimulating hormone (TSH), a glycoprotein of 28 kDa. Thyroid stimulating hormone is synthesized and secreted by anterior pituitary cells called thyrotrophs. Thyroid stimulating hormone exerts its effects after specific binding to a transmembrane receptor (TSH-R) expressed by the follicular epithelial cells. Such binding activates a receptor-linked adenyl cyclase producing increased cAMP levels which in turn trigger intracellular production of T3 and T4. Although several factors including hormones and neurotransmitters, influence TSH production and secretion, the levels of TSH are predominantly regulated by a hypothalamic-derived factor called thyrotrophin releasing hormone (TRH). Thyrotrophin releasing hormone is known to bind with high affinity to specific receptors on thyrotrophs stimulating TSH production which subsequently triggers biosynthesis and release of thyroid hormones. The circulatory levels of T3 and T4 in turn exert negative feedback control on TSH production by virtue of their action on pituitary thyrotrophs (P. R. Larsen, 1982).

1.1.2 Autoimmunity and the thyroid

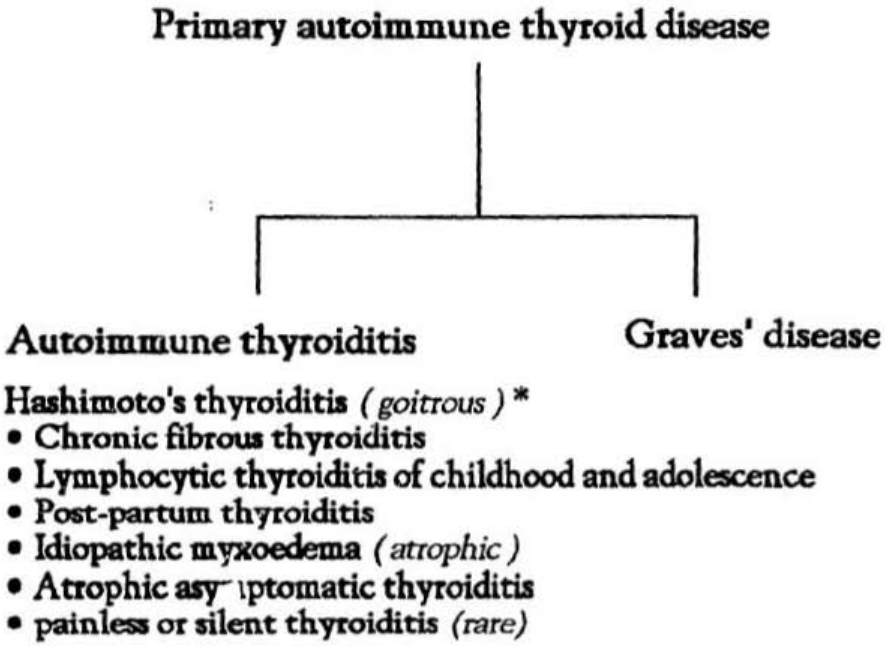
1.1.2.1 Autoimmunity and autoimmune thyroid disease

The term " autoimmunity " is used to describe an immune response directed to "self" constituents of an organism. According to the clonal selection theory, a primary function of the immune system is self non-self discrimination (F. M. Burnet, 1969). Burnet proposed that while B and T lymphocytes specific for foreign antigens are selected to survive and mature to immunocompetent cells, self-reactive lymphocytes are eliminated during their ontogeny via a process called clonal deletion (F. M. Burnet,

1959). Recent advances in immunology, however, have suggested autoreactivity to be a normal feature of the immune system as autoreactive clones are detected not only in patients but also in healthy individuals (S. Avrameas, 1991). What keeps those autoreactive clones from being autoaggressive? In other words what regulates the autoreactive clones and prevents them from attacking self tissues? It is suggested that a regulatory network exists within an organism that controls autoreactivity (L. R. Cohen & D. B. Young, 1991; S. Avrameas, 1991). Disturbance of that fine balance, due for example to environmental triggers such as infection, leads to uncontrolled autoreactivity that is expressed in the form of an autoimmune disorder (I. R. Cohen, 1991; L. R. Cohen & D. B. Young, 1991).

Autoimmune disorders are classified as systemic or organ-specific depending on distribution of the antigen towards which the autoimmune response is directed, and the number of tissues or organs in which autoaggression is observed (I. M. Roitt *et al.*, 1992). Thyroid autoimmune disorders belong to the second category because the immune response is directed to antigens within the thyroid and the lesions are restricted to the same organ. Autoimmune thyroid diseases are defined as primary or secondary depending upon the existence of lymphocytes in the peripheral blood which are specific for thyroid antigens. Primary thyroid disorders involve Hashimoto's thyroiditis (HT) and its variants and Graves' disease (GD) (Table 1.1). These two disorders have opposite manifestations in humans. HT is characterized by hypothyroidism, high levels of TSH and low levels of thyroid hormones in the periphery. GD is characterized by hyperthyroidism, low levels of circulatory TSH and high levels of thyroid hormones. In spite of differences in their clinical appearance the two disorders share common features (reviewed in J. Charreire, 1989). For example, both diseases aggregate in the same families or even coexist in the same thyroid (reviewed in R. Volpé,

Table 1.1: Classification of primary autoimmune thyroid diseases (R. Volpè, 1990).



* prototype

• variants

1990). Similarly progression from one thyroid condition to the other has been reported. Lymphocytic infiltration and antibodies specific for Tg and thyroid peroxidase (TPO) are observed in both HT and GD (reviewed in R. Volpé, 1990). Based on such features it has been suggested that the underlying autoimmune process is similar in both thyroid disorders (L. J. DeGroot & J. Quintans, 1989). However, other investigators consider them as separate but closely related autoimmune disorders mainly based on differences in other genetic, immunologic, and clinical features (reviewed in R. Volpé, 1990). For example, thyrotropin receptor antibodies are found in virtually all GD patients but they are variably present in HT patients. Similarly, in cases where HT and GD occur in the same families they tend to associate with the same haplotypes but when large populations of HT and GD patients are studied the two thyroid disorders appear to associate with different human leukocyte antigen (HLA) haplotypes (reviewed in R. Volpé, 1990). For example, in Caucasians GD has been found to be associated with DR3 and HT with DR5. HT has mostly been studied through the induction of an analogue of the disease in mice (EAT). An overview of HT follows.

1.1.2.2 Hashimoto's thyroiditis

The term "*struma lymphomatosa*" was initially used in 1912 by a surgeon to characterize the thyroid condition of four middle aged women with diffuse and massive infiltration of the thyroid gland (reviewed in P. E. Bigazzi & N. R. Rose, 1985). The same thyroid condition was later called HT although the term is currently used generally to describe the goitrous form of autoimmune thyroiditis. Several other synonyms such as chronic thyroiditis, lymphocytic thyroiditis, lymphadenoid goiter and autoimmune thyroiditis have been used to refer to the same pathologic condition (L. J. DeGroot *et al.*,

1984b). The term autoimmune thyroiditis is relatively broad, encompassing not only HT but also several other less common variants which are slightly different in their clinical appearance from the prototype (Table 1.1), (R. Volpé *et al.*, 1990).

Although HT affects all ages and both sexes, it is observed 9-25 times more frequently in women than in men (I. R. McDougall, 1992b). The peak of disease incidence is observed between 30 and 60 years of age although a juvenile variant occurs in children and adolescents (Table 1.1), (R. Volpé *et al.*, 1973).

Clinically the disease is characterized by the presence of diffuse, painless enlargement of the thyroid gland (thyroid gland weight ranges between 25-250 g), (V. A. LiVolsi, 1990) which is associated with euthyroidism, hypothyroidism or even in rare occasions with hyperthyroidism (see section 1.1.1.2), (reviewed in P. E. Bigazzi & N. R. Rose, 1985). Evidence of humoral immunity to thyroid antigens is also observed accompanied by characteristic histological changes of the thyroid.

The autoimmune nature of the disease was initially demonstrated in 1956 by Roitt *et al.* who were able to precipitate immunoglobulins from HT patients' sera reactive to thyroid antigens and Tg (I. M. Roitt *et al.*, 1956). Approximately 40 years later, the etiology of the disease is still obscure and the precise antigen involved in autosensitization of lymphocytes is undefined (R. Volpé, 1991). Candidates involved in this process include Tg and TPO, the principal thyroid antigens to which most of the antibody response is directed.

Serology: Antithyroglobulin (anti-Tg) antibodies have been demonstrated in the sera of HT patients by a variety of methods such as indirect immunofluorescence, passive hemagglutination, radioimmunoassay and enzyme-linked immunosorbent assay (ELISA). Depending on the sensitivity of the method employed, Tg-specific antibodies have been found in 55-90% of HT patients, but the antibody levels do not correlate well with

thyroid pathology (P. E. Bigazzi & N. R. Rose, 1985). Thus, the presence of anti-Tg antibodies is not indicative of HT since such antibodies have been observed in patients with other thyroid disorders of autoimmune or non-autoimmune origin (GD, multinodular goiter, thyroid carcinoma) as well as in 38% of healthy euthyroid individuals (P. E. Bigazzi & N. R. Rose, 1985; T. Kohno *et al.*, 1988). In addition, several HT cases have been reported in the absence of circulatory anti-Tg antibodies (J. R. Baker *et al.*, 1988). Conversely, anti-thyroid peroxidase (anti-TPO) antibodies have been found in virtually all HT patients, in most of those with GD, and less frequently in those with other thyroid disorders (R. Volpé, 1991). A close association also exists between anti-TPO antibody titers and histologic lesions of HT patients (H. Yoshida *et al.*, 1978). In addition many patients with thyroid autoimmunity lack Tg-specific antibodies in the periphery, but have TPO-specific antibodies which closely relate to their thyroid abnormalities (A. R. Tanner *et al.*, 1982). This suggests that TPO is the principal initiating autoantigen in human thyroiditis (H. Yoshida *et al.*, 1978; reviewed in R. Volpé, 1990). Although the importance of Tg in the initiation of human thyroiditis is not conclusive it should not be underestimated for several reasons. First, in HT patients seronegative for anti-Tg antibodies, it has been proposed that Tg-specific antibody production is localized within the thyroid (S. R. Baker *et al.*, 1988). Second, an initial autosensitization to Tg might be followed by anti-TPO antibody production. This concept is supported by Rose who detected anti-TPO antibodies after immunizing animals with Tg (J. A. Andrada *et al.*, 1968; N. R. Rose, 1988). Third, Tg and TPO were shown to express common epitopes for both B (Y. Kohno *et al.*, 1988; J. Ruf *et al.*, 1992) and T cells (S. M. McLachlan & B. Rapoport, 1989; A. Hoshioka, 1993). In other words TPO-specific monoclonal antibodies (MoAbs) cross-reacted strongly with Tg and vice versa (Y. Kohno *et al.*, 1988). Similarly, LNC derived from animals

immunized with the Tg-P4 (2730-2743) peptide responded significantly to TPO and TPO-P4 (118-131) peptide (A. Hoshioka, 1993). Fourth, the principal triggering event in the disease cascade could be based on Tg-specific T-cell reactivity with the antibody having a marginal role in the disease process. Evidence from experimental models supports such a notion (see section 1.2.3.1). Finally in both spontaneous and experimental models of thyroiditis, Tg was shown to be the principal autoantigen for the disease etiopathogenesis (reviewed in G. Wick *et al.*, 1986).

Although most of the antibody response is directed toward Tg and TPO, antibodies to other thyroid antigens, such as thyroid hormones (reviewed in R. Volpé, 1990), second colloid antigen (B. M. Balfour *et al.*, 1961), a new autoimmune thyroid disease related-antigen (ARTA-1) (H. Hirayu *et al.*, 1987) and other undefined thyroid surface antigens have occasionally been observed (reviewed in R. Volpé, 1990). In addition, there have been reports of HT patients whose sera were characterized by the presence antibodies with thyroid growth stimulatory (H. A. Drexhage *et al.*, 1980) or inhibitory antibodies (H. A. Drexhage *et al.*, 1981) that might be responsible for the goitrous and atrophic features respectively of autoimmune thyroiditis .

Histopathology: Histologically the thyroid gland exhibits invasion by mononuclear cells and the accumulation of these cells leads to disruption of thyroid follicles and damages the follicular basement membrane. Occasionally, characteristic germinal centers similar to those of secondary lymphoid organs are formed due to accumulation within the follicular lumen of mainly B lymphocytes but also T lymphocytes and macrophages (reviewed in P. E. Bigazzi & N. R. Rose, 1985). Thyroid follicles change from normal to necrotic and gradually the thyroid parenchyma undergoes fibrosis. Occasionally the thyroid epithelial cells appear swollen or oxyphilic due to accumulation of numerous large-size mitochondria. Such cells are known as Hurthle or Askanazy's

cells. Since the thyroid gland is slowly damaged, a part of the thyroid parenchyma cannot contribute to thyroid hormone synthesis and secretion. If the damage is extensive the patient expresses hypothyroidism. If, on the other hand, the thyroid gland destruction is not extensive, hypothyroidism is not observed. In such cases, the gland homeostasis is retained through the "healthy" functional part of the gland which compensates with overproduction of thyroid hormones under excessive TSH stimulation (see section 1.1.1.4), (reviewed in P. Laurberg, 1990). In some patients, hyperthyroidism is observed as a result of the coexistence of autoimmune thyroiditis and GD within the same thyroid gland. Such a condition is known by the term *"Hashitoxicosis"* and is due to the presence of anti-thyroid stimulating hormone receptor (anti-TSH-R) antibodies that mimic the TSH effect (see section 1.1.1.4), (reviewed in R. Volpé, 1990). Anti-TSH-R antibodies have been detected in approximately 26% of HT patients (S. Atkinson *et al.*, 1988). Their net effect on the thyroid clinical picture depends on the thyroid follicular integrity since extensive thyroid damage abrogates hyperthyroidism (S. Atkinson *et al.*, 1988).

HT is associated with other endocrine and non-endocrine autoimmune diseases. More common endocrine associations include, GD, insulin-dependent diabetes mellitus (IDDM) and Addison's disease (autoimmune adrenalitis). The most common non-endocrine diseases associated with HT include pernicious anemia, vitiligo, myasthenia gravis and Sjogren's syndrome (reviewed in R. Volpé, 1990). The reason for such associations is presently unknown. It is proposed that coexisting diseases might share similar etiologies as, for instance, a defect in organ-specific suppressor T cells (reviewed in R. Volpé, 1990). Such a defect has been implicated as an etiologic factor in both HT (reviewed in R. Volpé, 1990) and IDDM (J. F. Bach, 1988), however, its relative importance in coincidental disorders such as those mentioned above remains elusive.

Alternatively, coexisting diseases might associate with particular HLA polymorphisms. For example, the form of IDDM that is related to autoimmune thyroid disease has been related to HLA-B8 and Dw3 (reviewed in R. Volpé, 1990).

Even though the etiology of HT is still undefined it is considered to be a multifactorial disease. Genetic, immunological and environmental risk factors have been implicated in its pathogenesis, part of which will be discussed further in the following sections. In the induced murine model of thyroiditis the disease is clearly T-cell mediated and the antibody role is less prominent but in human thyroiditis the individual contribution of humoral and cellular factors in the disease process are still unresolved. Several immunological mechanisms have been proposed to operate in thyroid gland histological changes. Those include direct cytolysis of thyrocytes by cytotoxic T-cells (G. W. Canonica *et al.*, 1986); antibody-dependent cell mediated cytotoxicity (ADCC) by natural killer (NK) cells via interaction with immunoglobulin (Ig) bound to thyrocytes, complement mediated cytotoxicity via formation of immune complexes on a target cell, or direct injury of the gland by cytokines released by the infiltrated thyroid (reviewed in A. P. Weetman, 1992). In spite of the proposed mechanisms the precise sequence of events leading to thyroid damage is still unknown.

1.1.3 Major thyroid autoantigens.

In primary autoimmune thyroid disorders most of the autoreactive response is directed to three cardinal thyroid specific antigens, Tg, TPO and TSH-R. Although autoreactivity has been demonstrated to all three autoantigens in both HT and GD, the etiopathogenic candidates of the former disorder are thought to be Tg, TPO and for the latter TSH-R. Autoreactive responses to other thyroid antigens such as the second

colloid antigen (B. M. Balfour *et al.*, 1961), ATRA-1 (H. Hirayu *et al.*, 1987), the 70 kDa (J. Y. C. Chan *et al.*, 1989) and 65 kDa antigens (Q. Dong *et al.*, 1991) have also been reported however their significance in thyroid autoimmunity is undefined. Since the present study focuses on Tg, its structure, cellular distribution and physiological role will be further discussed.

1.1.3.1 Thyroglobulin

Tg is a large homodimeric glycoprotein of a molecular mass of 660 kDa and sedimentation coefficient of 19S. It is composed of two 12S identical subunits, 330 kDa each, joined through covalent (disulfide bond) or non-covalent linkage (reviewed in G. Medeiros-Neto *et al.*, 1993). The ability of Tg subunits to associate firmly via disulfide bonds depends on their iodination level. Poorly iodinated Tg easily dissociates into 12S subunits because the number of inter-chain disulfide bonds is low (reviewed in U. Bjorkman & R. Ekholm, 1990).

Tg is synthesized abundantly on rough endoplasmic reticulum polysomes by thyroid follicular cells and is subsequently transported to the Golgi apparatus where it undergoes postranslational modifications such as glycosylation and phophorylation. Finally the molecule is secreted into the follicular lumen for iodination and storage. Tg composes approximately 75-80% of total thyroid protein although the amount that is present in the thyroid at any time depends on the functional status of the gland. Thus, the hyperactive thyroid gland contains small deposits of Tg (A. J. Van Herle *et al.*, 1979a; reviewed in U. Bjorkman & R. Ekholm, 1990).

Tg serves two fundamental purposes necessary for normal thyroid gland function. First, within its protein matrix are incorporated tyrosine residues that facilitate iodine

trapping. Following iodination the Tg tyrosyl residues form monoiodotyrosines (MIT) and diiodotyrosines (DIT) that participate in the coupling reaction which results in synthesis of thyroid hormones. Second, stored Tg consists of a pool of iodine and bound thyroid hormones which are available for use upon demand (reviewed in G. Medeiros-Neto *et al.*, 1993).

Based on recombinant DNA technology it has been possible to derive the amino acid (a.a.) sequence of human Tg (Y. Malthièry & S. Lissitzky, 1987), bovine Tg (L. Mercken *et al.*, 1985) and part of rat Tg (R. DiLauro *et al.*, 1985) from messager RNA (mRNA). However, the mouse Tg (MTg) sequence is still undefined. High homology between the primary structure of Tg from various species is observed. For example human and bovine Tg exhibit 81% homology at the mRNA level (reviewed in G. Medeiros-Neto *et al.*, 1993).

Analysis of the primary a.a. Tg sequence reveals internal homologies. Based on those homologies, human Tg (HTg) is subdivided in four domains. The first internal homology is a 10 (times) repeat of approximately 50 a.a. positioned in the A domain between a.a. 29 and 1196. The second internal homology comprises 3 repeats of 15-17 residues each, localized in domain B of Tg between a.a 1436 and 1483. Finally the last internal homology is situated in domain C of Tg and consists of a motif existing in two subtypes and repeated five times between residues 1603 and 2186 (Y. Malthièry & S. Lissitzky, 1987). The carboxy-terminal of Tg corresponds to domain D. It is composed of approximately 600 a.a. and exhibits no internal homology (reviewed in G. Medeiros-Neto *et al.*, 1993). However it shows sequence similarity (28%) with about 90% of the sequence of acetylcholinesterases from both vertebrates and invertebrates (J. T. Dunn, 1991). Bovine Tg follows a similar pattern of internal homologies.

Tg can trap, via tyrosine iodination, 90-95% of the iodine that is available for protein binding (L. J. DeGroot *et al.*, 1984c). Although it contains about 140 tyrosine residues per mole, only 40 tyrosine residues are iodinated at an iodination level of 1% (reviewed in U. Bjorkman & R. Ekholm, 1990). The iodine content of Tg *in vivo* varies depending on the iodine intake by the thyroid, the species and the physiological condition. In most mammals, it ranges from 0.2 to 1% per molecule which corresponds to 10-50 atoms of iodine per mole of Tg. The normal physiological range of Tg-iodine content in humans is 0.25-0.5% per molecule (reviewed in U. Bjorkman & R. Ekholm, 1990). The distribution of iodinated tyrosines on Tg varies with the level of iodination. Among the iodinated tyrosines only a small part contributes to thyroid hormone synthesis, e. g. in a 0.5% iodinated Tg molecule no more than one T4 residue is present per molecule and T3 is present only in few of them. In cases of poor Tg iodination where the iodine content is less than 0.1%, no T4 or T3 is present per Tg molecule (L. J. DeGroot *et al.*, 1984c). In HTg, four acceptor hormonogenic sites (sites converted to thyroid hormones) have been demonstrated and are localized at the amino and carboxy termini of the molecule, a feature that makes them easily accessible to thyroid proteases. The hormonogenic sites have been mapped at positions 5, 2553, 2567 and 2746 of HTg (Y. Malthièry & S. Lissitzky, 1987). Although no hormonogenic site has been observed at the central part of the Tg molecule, it has been proposed that this portion influences the Tg structure and is therefore extremely important for thyroid hormone formation.

Ten per cent of thyroglobulin's weight is made up of carbohydrates. All species encompass two carbohydrate units, the polymannose chain and the complex chain. The polymannose chain contains two N-acetylglucosamine residues and 5-9 mannose residues that are linked to asparagine. The complex chain is also attached to an asparagine residue and is composed of a polymannose chain with three mannose residues

and various numbers of other peripheral sugars such as galactose, N-acetylglucosamine, sialic acid and fucose (reviewed in U. Bjorkman & R. Ekholm, 1990). HTg contains two additional carbohydrate units that are linked to the Tg core via serine or threonine residues.

Low circulating Tg- levels ranging from 1 to 30 ng/mL have been detected in the sera of many normal individuals. It is suggested that trace amounts of Tg, less than 1 ng/mL and practically undetectable with current radioimmunoassays are present in virtually all healthy subjects (A. J. Van Herle *et al.*, 1973). A rise in the circulatory levels of Tg however was demonstrated in cases of autoimmune thyroid disease or other thyroid dysfunction such as thyroid carcinoma, subacute thyroiditis, etc. (G. Torrigiani *et al.*, 1969). In addition, increased levels of Tg were detected in the bloodstream of neonates and Tg- binding lymphocytes were present in healthy subjects before birth, suggestive of the ability of T and B lymphocytes to tolerize to Tg in early life (A. J. Van Herle *et al.*, 1973; reviewed in A. J. Van Herle, 1990; I. M. Roberts *et al.*, 1973). Similarly based on sensitive assays, Tg-specific antibodies were detected in healthy individuals (36% women and 15% men), (reviewed in L. J. DeGroot & J. Quintans, 1989). Moreover, B cells derived from control subjects were triggered to secrete Tg-specific antibodies *in vitro* suggesting that a low level of autoreactivity to Tg, ("normal autoreactivity"), exists in healthy subjects.

1.2 EXPERIMENTAL THYROID AUTOIMMUNITY- AN OVERVIEW

1.2.1 Animal models of autoimmune thyroiditis.

In an attempt to design preventive and therapeutic strategies, early studies seeking to elucidate the mechanisms underlying the pathogenesis of autoimmune thyroid disease, were focused on animal models. Although animal models can only mimic the human autoimmune condition to a certain extent, they are advantageous relative to their human counterparts for several reasons. First, they provide a system where both the immunologic and pathogenic features of thyroid disease can be studied under controlled conditions. They can be manipulated by experimental procedures that cannot be employed in humans such as thymectomy, thyroidectomy, adoptive transfers of serum or cells etc. They include species with short life spans so that the natural history of the autoimmune process can be easily followed and detailed genetic studies can be easily performed. Finally they are composed of inbred populations and, in experiments, large numbers of animals allow valid statistical analysis.

No representative experimental model has been reported to date in GD but a variety of both spontaneous and induced experimental models exist for HT. Spontaneous thyroiditis arises naturally without requirement of any experimental manipulation, thus its etiopathogenic factor (s) is (are) unknown. Attempts to discern such factor(s) can be hindered by the long interval that might intervene between the disease triggering event and the full expression of its clinical signs. Induced thyroiditis, on the other hand, develops after specific *in vivo* experimental manipulation. Induced models of thyroiditis constitute more artificial analogues of human thyroid disease compared to spontaneous models, however, they allow one to initiate the autoimmune process at a certain time

point and follow it from that point through its entire course. Regardless of their nature (spontaneous or experimental) animal models of autoimmune thyroiditis contribute considerably to our understanding of the risk factors involved in thyroid autoimmunity (see following sections).

1.2.1.1 Spontaneous animal models of thyroiditis

Spontaneous autoimmune thyroiditis (SAT) has been observed in several animal species including chickens (reviewed in G. Wick *et al.*, 1989), rats (A. Hajdu & G. Rona, 1969 ; E. Sternthal *et al.*, 1981), dogs (W. E. Tucker & V. C. Captain, 1962 ; W. H. Beierwaltes & R. H. Nishiyama, 1968), monkeys (reviewed in P. E. Bigazzi & N. R. Rose, 1975) mice (N. F. Bernard *et al.*, 1991) and mastomys (a desert rodent) (H. A. Solleveld *et al.*, 1982a).

Chickens: The best studied spontaneous model of thyroiditis that closely resembles the human autoimmune disorder, in clinical, histopathological, endocrinological and immunological aspects, is the obese strain (OS) chicken (reviewed in G. Wick *et al.*, 1985). OS chicken is a white Leghorn line derived from hypothyroid Cornell strain (CS) chickens by selective breeding. Initially hypothyroidism was observed in a small core (<1%) of female CS chickens (R. K. Cole, 1966). After about 14 generations of breeding the hypothyroid characteristics were expanded within the flock to such an extend that approximately 100% of chickens exhibited severe hypothyroidism at the age of 3-5 weeks regardless of their sex. Apart from hypothyroidism other additional features of OS chickens include small body size, small combs, long silky feathers, lipaemic serum, large subcutaneous and abdominal deposits of fat, sensitivity to temperatures $< 20^{0}$ C and low fertility and hatchability.

Histologically OS chickens exhibit a thyroid picture very similar to that of HT patients, characterized by mononuclear infiltration of the thyroid by plasma cells, small and large lymphoid cells and macrophages. B cells are often distributed between thyroid follicles and acinar cells, a feature known as "peripolesis". Although characteristic germinal centers are observed, no Hurthle, giant cells or granulocytes are present in their thyroids. Usually infiltration starts multifocally 1-2 weeks after hatching and expands considerably leading to complete disruption of the thyroid follicular architecture after 3 weeks when the hypothyroid condition is prominent. In advanced cases of hypothyroidism some parts of the thyroids are characterized by fibrosis due to proliferation of the connective tissue.

Serologically 90% of OS chickens are characterized by the presence of antibodies to thyroid antigens including Tg (>65%), thyroid hormones and microsomal antigen (26%). However no antibodies to the second colloid antigen are observed (G. Wick *et al.*, 1989; P. E. Bigazzi & N. R. Rose, 1975; G. Aichinger *et al.*, 1984). Antibodies to other non-thyroid autoantigens have also been demonstrated in the serum of OS chickens (G. Aichinger *et al.*, 1984) however, no lesions in other organs have been reported.

Even though several spontaneous models of autoimmune thyroiditis exist, the OS chicken model is the best studied to date for several reasons. It is the only model where severe hypothyroidism develops that closely resembles the human disorder because it develops naturally in the absence of any experimental manipulation (reviewed in W. Wick *et al.*, 1989). In contrast to other models of thyroiditis almost all animals develop thyroid autoimmunity. Diseased animals can be clearly identified within the flock because they exhibit overt clinical signs of hypothyroidism such as small body size, fat deposits, etc. In birds there is a clear distinction between the B- and T-cell

compartment, therefore their immune system can be easily manipulated. Avian erythrocytes are nucleated constituting a convenient source of DNA for molecular studies (G. Wick *et al.*, 1989). The chicken embryo, once extramaternal, can be accessed easily. A large number of offspring derived from the same parents are available, facilitating statistical analysis.

Rats: There are two spontaneous models of autoimmune thyroiditis in rats. The first model was identified in 1969 by Hajdu and Rona who observed that inbred Buffalo (BUF) male rats at 36 weeks of age exhibited spontaneous histological changes of their thyroids comparable to those of HT (A. Hajdu & G. Rona, 1969). Thyroid lesions were characterized by the presence of lymphocytes, plasma cells and macrophages. Similarly to OS chickens, germinal centers were prominent within their thyroids whereas Hurthle cells were absent. Further studies indicated that the histological alterations of BUF rats were associated with a hypothyroid condition estimated by elevated and decreased levels of TSH and T4 respectively (J. D. Kieffer *et al.*, 1978).

The incidence of the disease was shown to be age-dependent rising from 14% at 12 wk of age to 48% at 30 wk (D. A. Silverman & N. R. Rose, 1971), and sex-dependent with a female: male ratio of 3:1 (B. Noble *et al.*, 1976). BUF rats appeared to be highly susceptible to the development of autoimmune thyroiditis because, under certain treatments such as subcutaneous injection with trypan blue, methylcholanthrene (chemical carcinogen), neonatal thymectomy etc., the disease incidence increased in young animals (P. E. Bigazzi & N. R. Rose, 1975; D. A. Silverman & N. R. Rose, 1974). Employing a variety of techniques such as indirect immunofluorescence and tanned cell hemagglutination tests, Tg-specific but not TPO-specific antibodies were detected in the serum of those animals with extensive thyroid lesions but not in those with mild or moderate thyroiditis (P. E. Bigazzi & N. R. Rose, 1975).

A second spontaneous model of autoimmune thyroiditis in rats is the Bio-breeding /Worcester (BB/W) model (E. Sternthal *et al.*, 1981). In addition to lymphocytic thyroiditis, BB/W rats exhibit infiltration of the pancreatic islets thus constituting a spontaneous model for IDDM. The incidence of autoimmune thyroiditis in this animal model is quite high (59%) at the age of 8-10 months in both sexes. The prevalence and intensity of the disease can vary considerably from 4.9% to 100% in different inbred sublines which are selected on the basis of IDDM pathogenesis, suggesting that the two hereditary autoimmune disorders are not tightly linked (R. Rajatanavin *et al.*, 1991). Regardless of the histological picture of the thyroid, estimation of the serum levels of T3, T4 and TSH in BB/W rats indicates normal values suggesting that thyroid infiltration by mononuclear cells is not extensive enough to result in thyroid failure (E. Sternthal *et al.*, 1981).

Dogs: Initial studies by Tucker *et al.* in 1962, demonstrated lymphocytic thyroiditis to develop spontaneously in 16.2% of beagle dogs. The disease was equally prevalent in males and females and, apart from the thyroid gland histological alterations, no other clinical signs of thyroid dysfunction were reported (W. E Tucker & V. C. Captain, 1962). Histological examination of the thyroids revealed scattered foci and diffuse infiltrates characterized by the presence of lymphocytes, plasma cells, macrophages and small numbers of neutrophils. Infiltrated cells were arranged in germinal centers and the presence of eosinophilic epithelial cells (Hurthle cells) was prominent. In most beagle dogs thyroid gland histological alterations were mild to moderate, thus resulting in normal thyroid gland function. However in those animals with severe thyroid gland changes, a decreased ^{131}I uptake was observed (W. H. Beierwaltes & R. H. Nishiyama, 1968).

Serologically, dog thyroiditis parallels the human disorder characterized by the presence of antibodies to Tg, microsomal and second colloid antigens (W. H. Beierwaltes & R. H. Nishiyama, 1968; G. J. Mizejewski *et al.*, 1971).

Monkeys: Marmoset monkeys of the genus *Callithrix* develop chronic thyroiditis (reviewed in P. E. Bigazzi & N. R. Rose, 1975). Monkeys of the genus *Saguinus* develop thyroiditis occasionally. Chronic thyroiditis in marmoset monkeys exhibits a female preponderance (female : male ratio 2:1). Thyroid glands are characterized by focal collections of inflammatory cells or total replacement of the follicular architecture by small and large lymphocytes and a few plasma cells. Follicles close to the infiltrated areas are atrophic, filled with very little or no colloid. Frequently mononuclear cells are present in the follicular lumen.

Serum derived from marmoset monkeys reacted weakly with rhesus monkey thyroid extract in hemagglutination assays (reviewed in P. E. Bigazzi & N. R. Rose, 1975).

Mice: An analogous model to the BB/W rat that spontaneously develops diabetes and lymphocytic thyroiditis was identified in mice. The NOD mouse exhibits infiltration of both Langerhans islets and thyroid within the 1st and 2nd month of life (N. F. Bernard *et al.*, 1991). The incidence and severity of the disease were shown to be age-dependent and equally prevalent in both sexes. No association of the disease with diabetes has been reported. Autoantibodies to thyroid antigens develop in 35% of the mice but are not reactive to Tg.

Preliminary studies have suggested that lymphocytic infiltration develops spontaneously in the thyroids of double transgenic mice that express the transgene for membrane-bound hen-egg lysozyme (HEL) under the control of rat Tg promoter and HEL specific T-cell receptor (TCR) (S. Akkaraju *et al.*, 1994). No signs of histological thyroid abnormality are observed in single HEL transgenic animals or in normal H-2^b

animals immunized with HEL or HEL peptides. In the same study, presence of auto-antibodies to thyroid specific antigens was not assessed.

Mastomys: Lymphocytic thyroiditis without any other clinical signs of hypothyroidism was observed in *Praomys natalensis*, a desert rodent commonly known as mastomys (H. A. Solleveld *et al.*, 1982a). The disease developed in 16% of males and 23% of females and was serologically characterized by the presence of auto-antibodies to second colloid antigen. Antibodies to Tg or TPO have not been reported (H. A. Solleveld *et al.*, 1982b). The thyroid infiltrate exhibited a diffuse or nodular pattern occasionally associated with either follicular epithelial cell hyperplasia or follicular distraction. Thyroid lesions were characterized by the presence of lymphocytes, plasma cells, and few macrophages. No clear-cut germinal centers were observed.

1.2.1.2 Induced models of autoimmune thyroiditis.

A step forward in the development of experimental thyroiditis were the experiments of Rose and Witebsky in 1956. Intradermal immunization of rabbits with isologous, autologous or heterologous thyroid extract in complete Freund's adjuvant (CFA) resulted in autosensitization and autoantibody production (E. Witebsky & N. R. Rose, 1956; E. Witebsky & N. R. Rose, 1958; K. L. Terplan *et al.*, 1960). Significant histological changes, corresponding to partial or extensive replacement of the thyroid tissue by infiltrating mononuclear cells, were also observed in the thyroids of the immunized animals (N. R. Rose & E. Witebsky, 1956). EAT was later induced in a variety of animal species such as guinea pigs (M. H. Flax, 1963), rats (R. S. Metzgar & J. T. Grace Jr., 1961 ; H. E. H. Jones & I. M. Roitt, 1961), monkeys (N. R. Rose *et al.*, 1966), dogs (K. L. Terplan *et al.*, 1960), chickens (B. D. Jankovic & K. Mitrovic, 1963)

and mice (R. S. Metzgar & J. T. Grace Jr., 1961; A. O. Vladutiu & N. R. Rose, 1972) by injection of homologous or heterologous thyroid extract in adjuvant. In those studies, the diagnosis of EAT was based on two criteria: the presence of mononuclear cell infiltration within the thyroid of immunized animals and the existence of autoantibodies specific for thyroid antigens. These two criteria, however, are not equivalent since existence of anti-thyroid antibodies did not correlate well with the thyroid pathology and, alternatively, thyroiditis could be observed in the absence of circulatory autoantibodies (reviewed in N. R. Rose *et al.*, 1965; reviewed in J. Charreire, 1989).

Direct approach of EAT induction: Subsequent studies were focused on the identification of the cardinal factor within the thyroid extract that triggers the autoimmune process. Initial observations in rabbits pointed to Tg (E. Witebsky *et al.*, 1958). As in the initial studies, and based on the same animal model of EAT, thyroiditis was induced after immunization of the animals with heterologous Tg or chemically modified homologous Tg in the absence of adjuvant (W. O. Weigle, 1965). Parallel observations in rats indicated that more severe thyroid histological alterations developed in immunized animals when a highly purified Tg fraction prepared by ultracentrifugation on sucrose density gradient was used for immunization instead of whole thyroid extract (I. M. Roitt *et al.*, 1965). The "light fraction" obtained through the same process, consisting of proteins with sedimentation constants less than 19S, and comprising microsomal and second colloid antigens, was not pathogenic (I. M. Roitt *et al.*, 1965). Analogous studies in mice indicated that thyroiditis develops after repeated immunization of the animals with soluble Tg (M. Eirehevy *et al.*, 1981). Although all of the above studies highlighted the principal role of Tg in the development of autoimmune thyroiditis, recent studies in mice suggest that purified and trypsinized porcine TPO (PTPO) (T. Kotani *et al.*, 1990) or the immunodominant PTPO peptide

which corresponds to 774-788 a.a. of PTPO, were also thyroiditogenic in H-2^b mice (T. Kotani *et al.*, 1992).

Thyroiditis via Tg immunization depends on the optimization of various parameters such as immunogenicity and quantity of the antigen (S. Shulman, 1971; J. Charreire, 1989), the route of antigen administration, the immunization schedule and the time of thyroiditis assessment.

Molecular modification of Tg: Various strategies have been employed in attempts to increase the immunogenicity of Tg, such as immunization with native Tg in adjuvant or immunization with both modified homologous or heterologous Tg either in a soluble form or emulsified in incomplete Freund's adjuvant (IFA) (W. O. Weigle, 1965). A variety of ways have been used for modification of homologous Tg such as heat-denaturation, enzymatic digestion and haptenization (W. O. Weigle, 1965; W. O. Weigle *et al.*, 1969). Such treatments terminate the natural tolerance of an organism to its own Tg, possibly via exposure of antigenic determinants of the molecule that are masked on native Tg and against which the T and B lymphocytes of the organism have not been tolerized. An additional factor that affects Tg immunogenicity resides in the procedure of Tg purification itself. Tg prepared by the ammonium-sulfate precipitation method was more potent in inducing autoantibodies and thyroid lesions in rabbits than that prepared by ultracentrifugation (N. R. Rose *et al.*, 1965). However, both Tg preparations were comparable in purity and identical immunochemically when assayed by agar electrophoresis and immunoelectrophoresis. The discordance of the above results has been attributed to changes in configuration that the Tg molecule might have undergone during purification by the ammonium-sulfate precipitation method and which might have rendered it more immunogenic.

Adjuvant: The nature of the adjuvant has also been shown to influence Tg immunogenicity. CFA (N. R. Rose *et al.*, 1971) and lipopolysacharide (LPS) (P. S. Esquivel *et al.*, 1977) were shown to be the most potent adjuvants in eliciting both anti-Tg antibodies and thyroid lesions in mice. The role of CFA in enhancing Tg immunogenicity could reside in its ability to produce a depot effect at the site of immunization where neutrophils and macrophages could be attracted and release their lysosomal enzymes, resulting in partial digestion of Tg. This process might increase its immunogenicity (W. O. Weigle, 1969). Alternatively, the adjuvant could alter the mode of antigen presentation resulting in disturbance in the clonal balance between autoantigen specific helper and suppressor cells (see section 1.2.3.1), (N. R. Rose & E. Taylor, 1991). Other adjuvants such as IFA (N. R. Rose *et al.*, 1965; F. J. Twarog *et al.*, 1970), silica (P. S. Esquivel *et al.*, 1977), alhydrogel (P. S. Esquivel *et al.*, 1977), pertussis vaccine (F. J. Twarog *et al.*, 1970) and alum (W. O. Weigle, 1965) were ineffective in inducing thyroiditis although some of those could elicit autoantibodies in various species, suggesting that the induction of autoantibody and thyroid lesions rely on distinct processes. The same conclusion was drawn in a recent study in mice, in which the natural tolerance to Tg was abrogated by conjugation of the molecule to class II MHC-specific MoAbs. Although this approach was successful in eliciting Tg-specific antibodies in genetically susceptible mice, possibly via a mechanism that targets the autoantigen on antigen-presenting cells (APC), it was ineffective in inducing thyroid lesions (B. Balasa & G. Carayanniotis, 1993a). An alternative approach for thyroiditis induction which bypasses the necessity for Tg purification, and the possible alteration in its structure which results in enhanced immunogenicity, was employed by Okayasu and Hatakeyama (1984). They produced thyroid lesions by implanting a fresh thyroid gland within the peritoneal cavity or under the kidney capsule of mice and followed this with

an intravenous injection of LPS. The induced autoimmune response was attributed to the combined effects of antigen leakage into the circulation originating from necrotic implanted thyroid tissue and polyclonal activation of B cells under the influence of LPS.

Tg-iodination: Both epidemiological and animal studies suggest that iodine content may enhance Tg immunogenicity (J. Charreire, 1989). Iodine-enriched Tg (containing at least 60 iodine atoms/molecule) obtained from CS chickens, when injected intravenously into normal birds in the absence of adjuvant, induced higher levels of anti-Tg antibodies than those observed following immunization with iodine-deficient Tg (<13 atoms of iodine/molecule), (R. S. Sundick *et al.*, 1987). Analogous studies in mice suggested that poorly iodinated Tg, although it could elicit antibodies in susceptible strains, could neither be recognized by Tg specific I-A^k-restricted T-cell hybridomas nor did it result in significant thyroid lesions (B. R. Champion *et al.*, 1987a). The mechanism by which the iodine content of Tg affects immunogenicity is not clear. It has been suggested that highly iodinated T-cell determinants of Tg are thyroiditogenic (B. R. Champion *et al.*, 1992; K. Dawe *et al.*, 1993). Alternatively, an increased level of Tg iodination might modify its processing by APC resulting in presentation of novel Tg epitopes that are not generated after processing of poorly iodinated Tg (B. R. Champion *et al.*, 1987a; G. Carayanniotis *et al.*, 1994).

The assessment time of established EAT is critical. Unlike HT, the disease is self-limiting in several species. Rats immunized with homologous or heterologous thyroid extract or purified Tg in CFA, developed thyroid lesions 2-4 weeks after immunization. Three to five months later no signs of inflammatory cells in the thyroid were observed (H. E. H. Jones & I. M. Roitt, 1961). Similarly, established EAT in a Rhesus monkey underwent regression to such an extent that almost no indication of infiltrate was visible within the thyroid 18 months after immunization and there was only a limited

degree of fibrosis. Therefore, absence of thyroiditis assessed at a certain time point does not assure inability of a particular immunization regimen to trigger the disease cascade.

Indirect approach of EAT induction: Apart from direct immunization of animals with Tg in adjuvant, an alternative approach to induce EAT is to increase the numbers of Tg-specific effector cells. Thus thyroiditis has been induced indirectly in guinea pigs (G. C. Sharp *et al.*, 1974; H. Braley-Mullen *et al.*, 1981), rabbits (R. M. Nakamura & W. O. Weigle, 1967), rats (F. J. Twarog & N. R. Rose, 1970) and mice (H. Braley-Mullen, 1985; L. L. Simon *et al.*, 1986) with transfer of spleen or lymph node cells (LNC) derived from animals actively immunized with Tg in CFA and activated *in vitro* with Tg or concanavalin A (con A) (I. Okayasu, 1985). Several studies attempted to isolate homogeneous populations of these effector cells and characterize them. Based on those studies, it was shown that EAT is a T-cell mediated disease since it could be adoptively transferred to naive recipients with Tg-specific T lymphocytes generated either *in vitro* by coculture with syngeneic thyroid epithelial cells (TEC) (J. Charreire & M. Michel-Bechet, 1982) or *in vivo* from animals with EAT (R. Maron *et al.*, 1983; C. G. Romball & W. O. Weigle, 1987).

Recent studies by Bruley-Mullen and colleagues have indicated that a severe form of EAT, called granulomatous thyroiditis, is induced in naive recipients by adoptive transfer of MTg-primed spleen cells activated *in vitro* in the presence of either anti-IL-2R or anti-IFN-γ MoAbs (H. Braley-Mullen *et al.*, 1991 ; S. J. Stull *et al.*, 1992). As in lymphocytic EAT, $CD4^+$ cells are required for the transfer of granulomatous EAT.

Although the previous studies suggested that T cells were the fundamental effector subset for mouse EAT, a recent study indicated that dendritic cells could also initiate and maintain the thyroid autoimmune process. Small numbers of dendritic cells (10^5 cells) exposed to Tg either *in vitro* or *in vivo* could initiate autoantibody production and

thyroid lesions in naive recipients (S. C. Knight *et al.*, 1988). Even though dendritic cells are not considered by themselves the effector subset for mouse EAT, they may provide a stimulus in the host for the production of autoreactive effector cells by presenting MTg continuously in the periphery or in the target organ (P. J. Kabel *et al.*, 1988) and therefore sustaining the autoimmune activation.

An alternative approach for inducing EAT is through manipulation of the T-cell subsets. Wistar rats subjected to thymectomy and whole body irradiation (5x200 rads) spontaneously developed both thyroiditis and anti-Tg antibodies (W. J. Penhale *et al.*, 1973). Typical HT lesions and Tg-specific antibodies also developed spontaneously in T-cell depleted mice after adoptive transfer of cells depleted in $CD5^{bright}$ T cells (S. Sugihara *et al.*, 1988). Subsequent cloning and characterization of the effector subsets revealed $CD5^{dull}$ $CD4^{+}$ T cells recognizing the antigen in the context of I-A (S. Sugihara *et al.*, 1993). Most of the clones were Tg-specific, supporting the concept of Tg as a principal autoantigen, although some of them were reactive to a yet unidentified thyroid component.

As stated previously, one of the advantages of animal models is the ability to manipulate their immune environment in ways that cannot be employed in humans. In the human condition, manipulation of immune system cells can only be performed in *in vitro* studies and these approaches may correlate poorly or not at all with the clinical condition. The availability of severe-combined immunodeficiency (SCID) and athymic "nude" mice, provided tools for the study of human-derived thyroid tissue and lymphocytes in an *in vivo* environment (reviewed in R. Volpé *et al.*, 1993). Both SCID and nude mice have certain features which enable them to accept xenogeneic grafts. SCID mice are characterized by lack of mature B and T cells due to a genetic defect in the function of the recombinase that is required for successful rearrangements of both

the Ig and the TCR genes (reviewed in M. A. Duchosal, 1992; reviewed in R. Volpè *et al.*, 1993). Therefore they can accept both lymphoid cells and thyroid grafts from human donors. Athymic nude mice can only accept human thyroid tissue xenografts because, although they lack mature T cells, they do express sufficient numbers of B and natural killer cells to lyse human lymphocytes (reviewed in R. Volpè *et al.*, 1993).

Among the models for induction of thyroiditis, the mouse model is the most extensively studied. It is an excellent model for immunogenetic studies due to the extensive characterization of its major histocompatibility complex (MHC) and the great availability of congenic, intra H-2-recombinant and congenic mutant strains. Mice can be easily handled and maintained in large numbers and at a lower cost than any other animal developing EAT. There is an enormous variety of mouse-specific reagents such as MoAbs specific for T- and B-cell surface markers which can be used in phenotypic and functional analyses of the cells involved in immune reactions. The generation time in mice is short, facilitating genetic studies (G. Wick *et al.*, 1981). For these reasons the mouse model was used in the current study.

1.2.2 Risk factors in thyroid autoimmunity

Both animal and human studies suggest that autoimmune thyroiditis is a multifactorial disorder. Early observations suggested that the disease is partly inherited because of its coincidence in monozygotic twins and its tendency to cluster in families (W. J. Irvine *et al.*, 1961; N. R. Farid, 1992). Because the coincidence rate of the disease among identical twins was less than 100% it was evident that other non-genetic factors also contributed to its pathogenesis. Therefore the disease was envisaged as the result of an interplay between genetic and environmental factors. Genetic factors play a

fundamental role because they are responsible for the dysregulation of the immune system as well as the susceptibility of the target organ. Environmental factors such as infectious agents and iodine intake are thought to act as initiating or precipitating agents leading genetically predisposed individuals to the development of thyroid autoimmunity (see following sections).

1.2.2.1 Genetic background

The study of genetic factors in human autoimmune thyroid disease is a difficult task because populations are heterogeneous and large family studies require several generations to complete. In addition, the frequency of the disease at the population level is low, therefore it is not easy to readily find significant numbers of patients to perform such studies. Thus the hereditary component of autoimmune thyroiditis has been mainly addressed in animal models of the disorder (C. L. Burek & H. S. Bresler, 1990). Such models have contributed considerably to our understanding of the number, the nature and localization of genes that might be implicated in the human disease. With respect to findings from both induced and spontaneous models of autoimmune thyroiditis, it is evident that the disease is multigenic and that both MHC and non-MHC genes contribute to its pathogenesis.

Current advances in the genetic aspects of experimental autoimmmune thyroiditis in mice will be discussed further in the next two sections. The genetic factors of spontaneous autoimmune thyroiditis in chickens, rats and humans will be addressed only briefly since they are beyond the scope of this study.

1.2.2.1.1 MHC genes

Mouse: The fundamental role of MHC genes in EAT susceptibility was highlighted by the pioneering work of Vladutiu and Rose, in 1971. Their study involved 33 inbred strains representing 11 different haplotypes in which EAT was induced by direct immunization with thyroid extract in CFA. Mice with different H-2 alleles were not classified as responders or non-responders but rather exhibited a graded degree of susceptibility. Mice carrying the H-$2^{k, s}$ haplotypes were designated as excellent responders, the H-2^{q} strains were good, strains with H-$2^{a, m, p}$ were fairly good, whereas H-$2^{b,d}$ and H-2^{v} were poor and very poor responders respectively. This classification of a given haplotype was based on the existence of mononuclear cell infiltration within the thyroid and the extent of its follicular destruction. Crosses between good (H-2^{s}) and poor (H-2^{d}) responders resulted in F1 hybrids that were also susceptible to EAT suggesting that the feature of susceptibility was transmitted to the progeny as an autosomal dominant trait (A. O. Vladutiu & N. R. Rose, 1971a). In the same study, congenic strains for the H-2 locus could be classified as good or poor responders depending on their H-2 type. For example, the congenic strains C3H.SW (H-2^{b}) and C3H/HeJ (H-2^{k}) characterized by the same background genes but different H-2 alleles, were classified as low and high responders respectively. Although the general pattern of susceptibility to EAT was confirmed by subsequent studies, deviations were occasionally observed. For instance the H-2^{q} haplotype represented by the B10.Q strain was later shown to exhibit a poor response to Tg although in the initial study of Vladutiu and Rose it had been designated as a good responder (K. W. Beisel *et al.*, 1982a; K. W. Beisel *et al.*, 1982b). The difference between initial and later results was attributed to

differences in the Tg dose, the nature of the adjuvant (LPS vs CFA) and other non-MHC gene influences (see next paragraph) (K. W. Beisel *et al.*, 1982b).

Vladutiu and Rose addressed the cellular basis of H-2-linked susceptibility to thyroiditis. They produced bone marrow chimeras by thymectomy, lethal irradiation and reconstitution of the animals with either B cells, T cells or a combination of both subsets (A. O. Vladutiu & N. R. Rose, 1975). They subsequently assessed the thyroid pathology of chimeras derived from susceptible (H-2^k, H-2^s) or resistant (H-2^d) strains following Tg immunization. Poor responders reconstituted with T lymphocytes either alone or in combination with B cells derived from high responders resulted in pathology indices markedly higher than the indices of animals reconstituted with poor responder T and B lymphocytes. These studies suggested that the H-2-linked susceptibility to thyroiditis is T-cell based. Analogous results were revealed by studies in which EAT was induced after adoptive transfer of effector cells into naive animals (see section 1.2.1.2). In those studies, the disease was transferrable to normal syngeneic recipients by MTg-primed T lymphocytes (H. Braley-Mullen *et al.*, 1985; I. Okayasu, 1985; W. V. Williams *et al.*, 1987), T-cell lines (R. Maron *et al.*, 1983) or clones (C. G. Romball & W. O. Weigle, 1987; S. Sugihara *et al.*, 1993) derived from donors carrying a susceptible H-2 genotype. Similarly the ability of normal T lymphocytes to be primarily sensitized by syngeneic thyroid epithelial cells and to transfer thyroiditis to normal syngeneic donors was dependent on their H-2 type (J. Salamero & J. Charreire, 1983a; J. Charreire J. & M. Michel-Bechet, 1982).

The MHC expression of the thyroid gland itself was shown to influence susceptibility to EAT, implicating a T-cell mediated damage of the target organ (A. Ben-Nun *et al.*, 1980). Susceptible (H-2^k x H-2^b) F1 hybrids implanted under the kidney capsule with thyroids that had originated from the susceptible parent (H-2^k) or resistant parent (H-

2^b) strains and subsequently injected with Tg in adjuvant, exhibited H-2 restriction of EAT susceptibility at the level of the implanted target organ. In other words, the incidence of lesions was high (87%) in implants derived from EAT susceptible H-2^k haplotype and low (20%) in those originated from EAT resistant H-2^b haplotype. Similar results were obtained by Okayasu using a different EAT model where the disease was induced by implantation of a thyroid gland under the kidney capsule and subsequent injection of the animals 6 hours later with LPS (I. Okayasu, 1986), (see section 1.2.1.2). In the same study, it was shown that the implanted thyroid gland, regardless of its origin (poor or high responder) produced Tg of the same immunogenicity. This last result contrasts with previous studies which reported that strain influenced the immunogenicity of Tg. Purified Tg derived from the congenic strains B10.D2 (H-2^d) and B10.Br (H-2^k) which are low and high responders respectively, differed in its ability to induce thyroid lesions and autoantibodies (V. Tomazic & N. R. Rose, 1976).

I-A region control. From the previous studies, it is evident that EAT induction in mice is under the genetic influences of MHC genes. Subsequent studies attempted to localize the responsible locus for EAT susceptibility within the H-2 complex. Preliminary reports based on intra-H-2 recombinant mouse strains which had been derived from recombinations among H-2^k, H-2^d and H-2^b haplotypes positioned the MTg response locus at the centromeric side of the H-2 region (V. Tomazic *et al.*, 1974). However, the lack of suitable intra-H-2-recombinant strains did not permit precise localization of the susceptibility gene(s). Further studies, based on new intra H-2 recombinant congenic strains of B10 background with various combinations of k, b, q alleles at the K and /or I-A regions, further localized the disease susceptibility to the I-A locus of the H-2 gene. This locus was called the Tg immune response gene (Ir-Tg) (K. W. Beisel *et al.*, 1982a). The significance of the I-A locus in EAT susceptibility was

further highlighted by the study of Vladutiu and Steinman who were able to completely prevent EAT induction in mice by treating the animals with anti-I-A MoAb either before or at the time of antigenic challenge with Tg in CFA. Treatment with anti-I-A MoAb after antigenic challenge could not completely prevent disease development, although it significantly reduced its severity (A. O. Vladutiu & L. Steinman, 1987). Similar results supporting localization of EAT susceptibility to the I-A locus were revealed by the study of Salamero and Charreire (1983b). These investigators developed a system in which lymphocytes could be sensitized *in vitro* (primarily sensitization) by thyroid epithelial cells. The primarily sensitized lymphocytes were characterized as $CD4^+$ and were restricted to the I-A locus of the H-2 complex.

D-region influences: Although the I-A locus is the major predisposing locus in the mouse EAT model, D and K genes can further modify its effect. Using four sets of recombinant strains which were expressing s, k, d, b alleles at the I-A locus and identical or different alleles at the K and D loci respectively, it was demonstrated that the D-end genes exert a regulatory effect on the disease process (Y. M. Kong *et al.*, 1979). However, the extent of this influence was highly dependent upon the origin of both Ir-Tg and K-end as well as D-end genes. For instance, when the Ir-Tg gene had originated from the good responder k and s strains and the I-2D-end gene from the d strain, both antibody levels and cellular infiltration were reduced. The greatest degree of thyroid inf..tration was observed when k or f alleles were expressed at the D-end locus. This suggests that an interaction among the I-A, D and K region gene products controls the outcome of the EAT phenotype. In the same study an attempt was made to study the modulatory effect of D-end genes using low responder strains of b and d haplotypes. However, in that case no clear-cut result could be discerned because the cellular

infiltration in low responder strains was minimal or nonexistent. It was suggested that the D-end gene(s) control(s) the effector mechanism of thyroid infiltration.

K-region influences: Genetic influences on EAT development were also attributed to the K-region of the H-2 complex. Initial studies by Maron and Cohen suggested an involvement of K-end genes in EAT incidence. Their conclusion was based on the use of the B6.H-2^{ba} (HZ1) strain of the b haplotype in which the H-2K locus has undergone a point mutation and instead of K^b expresses K^{ba}. Interestingly, it was shown that the point mutation at the H-2K gene transformed a low responder strain to a high responder. Thus, HZ1 mutants developed thyroiditis with an incidence similar to that observed in the high responder C3H (H-2^k) mice (R. Maron & I. R. Cohen, 1979). Subsequent studies by Beisel and colleagues re-examined a possible role of K-end genes in EAT induction using a series of H-$2K^b$ mutants. It was shown that mutational differences at the K end gene in the absence of the high responder Ir-Tg (I-A^k) gene in the I-A subregion of the H-2 complex had no effect on EAT susceptibility (K. W. Beisel *et al.*, 1982a). However, such an effect was clearly evident in strains carrying the high responder k allele at the I-A locus of the H-2 complex. For instance the percentage of animals with thyroid lesions in B10.A (k k k d) and B10.AQR (q k k d) was 52% and 70% respectively implying modulation of the disease incidence by K-end genes (K. W. Beisel *et al.*, 1982a; reviewed in R. C. Kuppers *et al.*, 1988).

The expression of H-2K in both thyroid gland and thymus was shown to be critical for the EAT phenotype (R. Maron & I. R. Cohen, 1980). The above conclusion was based on experiments in which either (B6.C-H-$2^{bm\text{-}1}$ x B6)F1 (A. Ben-Nun *et al.*, 1980) or (B6.H-2^{ba} x B6)F1 hybrids (R. Maron & I. R. Cohen, 1980) received under their kidney capsules thyroid gland implants derived from their respective parental strains. Infiltration of the implanted target organ was dependent upon its expression of the H-2K

allele, implicating cytotoxic T cells in the effector phase of EAT. Similarly, implantation of donor irradiated thymus in nude recipient (C_3H/eB x B6) F1 mice and subsequent immunization with Tg in adjuvant resulted in severe EAT only in the case where the grafted thymus had originated from the high responder strain (R. Maron & I. R. Cohen, 1980).

Rat: The contribution of genetic factors in the rat model of EAT is more complex and less understood than in the mouse model. From 13 inbred rat strains studied, no clear association between the RT1 locus (MHC genes in rats) and EAT susceptibility has been revealed (H. S. Lillehoj & N. R. Rose, 1982). However, in recent studies it has been shown that MHC genes contribute substantially to the final outcome of the disease. Using congenic PVG rats that differ only at the RT1 locus, it was demonstrated that animals carrying the $RT1^c$ haplotype were high responders whereas $RT1^u$ and $RT1^a$ were poor responders (H. J. De Assis-Paiva *et al.*, 1989).

Chicken: SAT in the obese strain chicken is a multigenic trait. Its pathogenesis is based on three defects that are controlled by at least three genes or gene complexes. Thus, a three-locus model has been proposed to explain the pattern of its inheritance (reviewed in R. C. Kuppers *et al.*, 1988 ; reviewed in G. Wick *et al.*, 1989). The first locus is MHC-associated and determines the immune response to Tg (M. D. Livezey *et al.*, 1981), whereas the other two are linked to non-MHC genes and are associated with abnormalities in the thymus and the thyroid giand (see section 1.2.2.1.2). Birds with the greatest incidence of SAT were shown to inherit all three loci, whereas those that carry only the MHC susceptibility locus exhibited only a minor degree of thyroiditis (G. Wick *et al.*, 1985). The relationship between the B locus (MHC genes in birds) and SAT was revealed by the study of Bacon and colleagues (L. D. Bacon *et al.*, 1974). Assessment of SAT development in homozygous birds carrying the B^{13}, B^5 or B^{15} haplotype revealed

extensive lymphoid infiltration in the thyroid glands of the B^{13} and B^{15} animals and mild in those with B^{5} (L. D. Bacon *et al.*, 1974; L. D. Bacon *et al.*, 1976). Those initial results were not verified by subsequent studies on an OS colony separated from the original flock for almost 10 years, suggesting the existence of non-MHC genes in the regulation of the SAT phenotype(reviewed in G. Wick *et al.*, 1989). Taken together, the studies show that the MHC-associated susceptibility in SAT is less clear than mouse EAT and no information exists as to where the susceptibility locus resides within the B region (reviewed in G. Wick *et al.*, 1989).

Human: In human thyroiditis, attempts to identify, within the HLA complex, an immune response gene similar to the H-2A region of murine EAT have been unfruitful. Although HLA associations with autoimmune thyroiditis have been observed, these appear to be weak and inconsistent in different populations (A. P. Weetman, 1992 ; N. R. Farid, 1992). Early studies revealed immunogenetic heterogeneity between the atrophic and goitrous variants of autoimmune thyroiditis (see table 1.1). For instance HT was associated with HLA-DR5 and primary myxoedema with HLA-DR3 (N. R. Farid *et al.*, 1981). In other studies, HT was associated with other alleles such as DR3 and DR4 (A. P. Weetman, 1992). All of these studies were based on the typing of haplotypes by serological analysis, a method whose sensitivity depends directly on the specificity of the typing reagents. Recent studies based on molecular typing by the restriction fragment length polymorphism (RFLP) method have revealed a strong association with the DQw7 specificity, which is encoded by the DQB1 and DQA1 genes rather than the DR locus (K. Badenhoop *et al.*, 1990). This last result was not confirmed in other studies in which HT was associated with DQw2 (N. Tandon *et al.*, 1991) or was not associated with any of the alleles at the DQ or DR loci (A. Mangklabruks *et al.*, 1991; D. Jenkins *et al.*, 1992). Since the relative risks associated

with HLA markers are low the inconsistency of the above results could be due to ethnic differences between the study populations.

What is the basis of an association between MHC and thyroiditis? It is well established that both MHC class I- and class II-encoded gene products play a critical role in T-lymphocyte activation. In both normal and autoimmune responses, T cells recognize short antigenic sequences associated with MHC gene products that are expressed on the surface of antigen presenting cells (reviewed in R. H. Schwartz, 1985). Due to MHC-restricted T-cell recognition, MHC gene products may participate in thyroid autoimmunity via two pathways. First, certain MHC class II products might favour the interaction with thyroiditogenic peptides. The resulting MHC-peptide complexes might in turn be recognized by pathogenic T cells. Second, MHC class II gene products expressed in the thymus might regulate the developmental selection (positive or negative) of thyroiditogenic T cells during T-cell differentiation and maturation in the thymus (reviewed in G. T. Nepom, 1991; reviewed in C. L. Burek & H. S. Bresler, 1990).

1.2.2.1.2 Non-MHC genes

The contribution of non-MHC genes to the development of thyroiditis has been assessed using congenic mouse strains carrying the same H-2 but different background genes and employing mild immunization protocols such as injection of the animals with MTg followed 3 hours later by LPS (K. W. Beisel *et al.*, 1982b). Significant influences of non-H-2 genes have been observed both on the severity and the incidence of thyroiditis as well as the levels of Tg-specific antibodies. For example, using the B10, BALB.B, C3H.SW and A.BY strains, all of which carry the H-2^b haplotype, the following

observations were made. First, strains carrying the C3H or BALB backgrounds exhibited higher autoimmune responses to thyroglobulin than B10 and A strains. Second, the highest antithyroglobulin titers and most severe lesions were found in the congenic strain of C3H background. Similar results were obtained when the B10.BR, BALB.K and C3H/Anf strains of the high responder H-2^k haplotype were compared. Other studies based on the use of recombinant inbred and congenic strains suggested that the Igh locus has an effect on the levels and the subclass distribution of anti-Tg antibodies (reviewed in R. C. Kuppers *et al.*, 1988). For example, following immunization of CBA-Tu (Igh^j) and CBA-Igh^b congenic strains with MTg in CFA it has been shown that the Igh^b haplotype produces minimal levels of IgG2a MTg-specific antibody compared to the Igh^j haplotype (reviewed in R. C. Kuppers *et al.*, 1988).

The most informative model in which the non-MHC effects have been studied is the OS model. In this animal model two additional factors contributing to SAT susceptibility have been identified (see section 1.2.2.1.1), over and above MHC-regulated immune responses. These are controlled by genes residing outside the B locus. The first set of genes affects the function of the thymus by altering the balance of helper and suppressor T-cell populations (reviewed in N. R. Rose *et al.*, 1980). It has been proposed that the thymus of the OS chicken undergoes an abnormal T-cell maturation. This becomes apparent as the effector cells are released in the periphery early by the OS thymus followed by a delayed release of the suppressor cells (reviewed in N. R. Rose *et al.*, 1980). Evidence for such a thymic abnormality comes from the experiments of Jankobisiak and colleagues who carried out transplantation experiments between B-locus matched normal or OS chickens (M. Jankobisiak *et al.*, 1976). Under those conditions skin graft rejection is due to differences at the minor histocompatibility antigens. According to those experiments neonatally thymectomized OS, but not normal chickens

exhibited an accelerated rejection of skin grafts. The last result suggested that in OS chickens the effector cells are released early to the periphery from the thymus. The second set of genes act on the target organ promoting its susceptibility to autoimmune attack. The first indication of the existence of a primary alteration of the target organ has been revealed from the experiments of Sundick and Wick (1974). The authors injected OS embryos and newly hatched chicks with ^{131}I and they estimated ^{131}I uptake by their thyroids 20 hr later. ^{131}I uptake in OS chicks was significantly higher than that in outbred New White Leghorn (NWL) chickens. A subsequent study by Sundick and colleagues proved that the elevated ^{131}I was an intrinsic property of the thyroid and not an outcome of differences in the levels of TSH between OS and normal chickens (R. S. Sundick *et al.*, 1979). To date the nature of the vulnerability of OS thyroid gland has not been determined. Apart from the increased rate of iodine uptake by the OS thyroid several other factors have been proposed. First, an altered iodine composition of Tg could increase the immunogenicity of the molecule (reviewed in G. Wick *et al.*, 1989; see section 1.2.1.2). Second, a primary aberrant expression of class II antigens by OS thyroid epithelial cells could trigger SAT development. Class II antigen expression by TEC in chickens is thought to be a secondary event of SAT induction because class II antigens are expressed only in the neighborhood of infiltrating T cells. The latter observation suggests that aberrant class II expression is induced by interferon γ (IFN-γ) that is secreted by T cells invading the thyroid (reviewed in G. Wick *et al.*, 1989). *In vitro* studies, however, have shown cultured TEC from OS chickens to have a lower threshold for the induction of MHC class II antigen expression with IFN-γ than cells from normal strains (reviewed in G. Wick *et al.*, 1989). Finally a viral infection might be responsible for the increased susceptibility of the OS thyroid to autoimmune attack. Evidence to support the latter possibility comes from recent studies showing that

OS chickens are characterized by the presence of an endogenous virus, ev22, not found in other strains tested (A. Ziemiecki *et al.*, 1988; reviewed in G. Wick *et al.*, 1989). Other genetic factors, x-linked genes for example, could also contribute to disease susceptibility (L. D. Bacon *et al.*, 1981).

1.2.3 CONTRIBUTION OF THE IMMUNE SYSTEM

Attempts to explore the individual contributions of humoral and cellular factors in EAT pathogenesis have been based mainly on two strategies. The first approach employs adoptive transfer of cells or serum from diseased donors to healthy animals, a procedure that can clearly discern the significance of cellular and humoral factors in EAT development. The other method looks for associations between Tg-specific T-cell responses or levels of Tg-specific antibodies and thyroid lesions, since such associations could offer clues to the potential involvement of T cells or antibody in the disease cascade.

Based on such methods, it was proposed that humoral and cellular factors combine to produce the final picture of autoimmune thyroiditis (reviewed in P. E. Bigazzi & N. R. Rose, 1985). However, the extent of their contribution to EAT pathogenicity varies, depending upon the species studied and the immunization procedure employed for thyroiditis induction (reviewed in A. O. Vladutiu, 1990). For example, in rabbits, close association between humoral autoimmune responses and thyroid lesions has been found (reviewed in P. E. Bigazzi & N. R. Rose, 1985). In contrast, in the rat, a significant correlation between thyroiditis and cell-mediated immunity has been reported (H. S. Lillehoj & N. R. Rose, 1982). In guinea pigs it has been suggested that cellular and humoral factors combine to modulate both the incidence and the severity of EAT (G. C.

Sharp *et al.*, 1974). Evidence coming from the mouse model of EAT suggests that the humoral or cellular contribution may vary depending upon the immunization regimen used for thyroiditis induction. Thus in thyroiditis induced by immunization with heterologous or self-altered Tg (haptenized, heat denatured or enzymatically digested Tg) it has been proposed that humoral factors have a predominant role but in EAT induced by immunization with Tg in adjuvant, T cells play a predominant role (C. G. Romball & W. O. Weigle, 1984).

1.2.3.1 T cells

Each organism normally responds strongly to invading foreign substances but exhibits an unresponsive state, or tolerance to its own constituents. In the past, self-tolerance was attributed to the clonal deletion of autoreactive immunocompetent cells (see paragraph 1.1.2.1). Since immune responsiveness to T-cell dependent antigens such as proteins requires the collaboration of both B- and T- cell subsets, the state of immunological tolerance to a self protein antigen could be achieved by clonal deletion of both B and T cells or T cells alone. Initial studies in autoimmune thyroiditis suggested that acquired immune unresponsiveness to Tg, an antigen found in relatively low levels in the circulation (G.Torrigiani *et al.*, 1969), was a result of deletion of Tg-specific T cells while the autoreactive B-cell subset remained unaffected (J. A. Clagget & W. O. Weigle, 1974). This view was further supported by two lines of evidence. First, Tg-reactive B cells (A. D. Bunkhurst *et al.*, 1973) and anti-Tg antibodies (T. Kohno at al., 1988; reviewed in S. Avrameas, 1991) were identified in healthy subjects. Second, the tolerant state of T-cell help could be overridden in mice using LPS together with the immunizing antigen (P. S. Esquivel *et al.*, 1977), or either foreign or altered-self Tg as

immunogens (W. O. Weigle, 1965a). In the first case, the requirement for T-cell help could be by-passed by polyclonal activation of the Tg-reactive B cells induced by LPS whereas in the latter case, B cells could receive help from cross-reactive T cells produced against altered Tg determinants. Subsequent evidence suggests that clonal deletion of Tg-reactive T cells was inadequate in explaining self-tolerance. Tg-reactive T cells not only exist in good responder mice (P. S. Esquivel *et al.*, 1978; M. Eirehewy *et al.*, 1981; S. Sugihara *et al.*, 1988) but also play a substantial role in the regulation of the autoimmune response (N. R. Rose *et al.*, 1981). Since both Tg-reactive B and T cells coexist in normal H-2 susceptible mice how is the tolerant state maintained? It was hypothesized that regulatory systems exist, such as T-suppressor circuits (Y. M. Kong *et al.*, 1982; N. R. Rose *et al.*, 1981) and idiotypic anti-idiotypic networks (M. Zanetti & P. E. Bigazzi, 1981; C. Roubaty *et al.*, 1990; B. Texier *et al.*, 1992a) and that these control the tolerant state (N. R. Rose *et al.*, 1980; N. R. Rose *et al.*, 1981; A. P. Weetman, 1992). The final outcome, abrogation of T-cell response or maintenance of immune unresponsiveness to Tg, depends on a fine balance between the cell populations that promote autoreactivity and those that control it (N. R. Rose *et al.*, 1981; G. Wick, 1985). Such regulatory mechanisms can be overridden in experimentally induced thyroiditis either by employing strong antigenic stimuli such as immunogens in adjuvants or by increasing the cell populations that mediate thyroiditis versus those that suppress it (see section 1.2.1.2). T cells appear to have a prominent role in EAT operating both as effector as well as regulatory cells.

Effector cells: Several lines of evidence both *in vivo* and *in vitro* are available to indicate that T cells play a critical role as effectors in murine EAT. First, as already discussed, the disease in mice is MHC-restricted and the cellular basis of that restriction has been attributed to the T-cell subset (see section 1.2.2.1.1). Second, direct evidence

supporting the critical role of T cells as effectors in EAT comes from adoptive transfer experiments (see section 1.2.1.2). Furthermore, thyroiditis was transferred successfully by T-cell lines to both irradiated (550 rad) and nude mice, strongly arguing against the participation of host B or T lymphocytes in the effector phase of the disease (R. Maron & I. R. Cohen, 1980). Similarly, transfer of mouse Tg-sensitized, B-cell depleted lymphocytes to either normal or B-cell depleted recipients resulted in thyroiditis. This further suggests that the disease does not require either MTg-primed B cells or B cells recruited from the host to develop (H. Braley-Mullen *et al.*, 1985; H. Braley-Mullen *et al.*, 1994). Third, nude mice actively immunized with either Tg emulsified in CFA (A. O. Vladutiu & N. R. Rose, 1975) or soluble Tg followed by LPS (P. S. Esquivel *et al.*, 1977) did not develop EAT. Fourth, *in vitro* proliferative responses of Tg-sensitized lymphocytes to Tg constitute an early marker of subsequent EAT development. Thus, in both mice and rats a close correlation was found to exist between the proliferative T-cell responses and susceptibility to thyroiditis (I. Okayasu *et al.*, 1981; H. S. Lillehoj & N. R. Rose, 1982; L. L. Simon *et al.*, 1985). Fifth, histological examination of mouse thyroid infiltrating lymphocytes, using membrane immunofluorescence to define the nature of the cell subsets included within the thyroid infiltrate, revealed T cells as the dominant subset. Although the numbers of T cells varied with time, they ranged from 27 to 50% throughout the second and third week after immunization (P. Creemers *et al.*, 1984). In contrast, B cells in the thyroid were constantly below 5% during the same period, in spite of their high levels (19-24%) in peripheral blood (P. Creemers *et al.*, 1984). Similar findings were revealed after *in situ* examination by tissue immunofluorescence of thyroid sections originating from diseased animals (D. Conaway *et al.*, 1987).

As soon as the importance of T cells in the induction of mouse thyroiditis was demonstrated, interest was directed towards the identification of the effector T-cell

subset(s) and elucidation of the mechanisms by which they mediate EAT. Such studies however, required the isolation, phenotypic characterization and functional analysis of the cell populations. The mouse model was the ideal animal system for those studies for two reasons. First, it was technically feasible to phenotypically characterize the different mouse T-cell subsets (see section 1.2.1.2). Second, the identification of cell growth factors and the advances in cellular cloning offered an opportunity to generate homogeneous mouse T-cell populations and retain them in culture for long periods. Taking advantage of the mouse system, investigators proceeded to isolate homogeneous Tg-specific T-cell populations by generating Tg-specific T-cell lines (R. Maron & I. R. Cohen, 1980; B. R. Champion *et al.*, 1985), clones (C. G. Romball & W. O. Weigle, 1987; S. Sugihara *et al.*, 1993) and hybridomas (J. J. Remy *et al.*, 1989; D. C. Rayner *et al.*, 1987). Phenotypic analysis and use of those populations in functional assays revealed that both helper and cytotoxic T cells participate in the effector phase of EAT. However, T-helper (Th) cells also play an essential role in the induction of thyroiditis. The contribution of Th cells as inducers /effectors was based on several observations. First, the *in vitro* Lyt1$^+$2$^-$ T-cell proliferative responses to MTg represent early markers of subsequent EAT induction (L. L. Simon *et al.*, 1985). Second, Sugihara and colleagues (1988) using adoptive transfer of T-cells depleted in a particular T-cell subset as a model of EAT, have shown that Lyt-1^{dull} L3T4^{bright} cells are required for thyroiditis development. Third, EAT has been transferred to naive animals by Th clones derived either from lymph node cells of actively immunized mice (C. G. Romball & W. O. Weigle, 1987; Y. Hiyama *et al.*, 1993) or from thyroid lesions of diseased animals (S. Sugihara *et al.*, 1993). Fourth, Lyt-1$^+$ cells have been found to be the predominant subset by either membrane immunofluorescence of thyroid infiltrating lymphocytes (P.

Creemers *et al.*, 1984) or by *in situ* examination of thyroid sections derived from mice with EAT (D. Conaway *et al.*, 1987).

These lines of evidence clearly indicate that Th cells participate in the effector phase of EAT, however, these are not the only subset involved. Evidence suggests that MTg-sensitized cells either *in vivo* (P. Creemers *et al.*, 1983; L. L. Simon *et al.*, 1986; Y. M. Kong *et al.*, 1986b) or *in vitro* (J. Salamero & J. Charreire, 1985) exhibit cytotoxicity for syngeneic thyroid monolayers. These cytotoxic cells were shown to be class I-restricted and they required the presence of Lyt-1$^+$ cells for their expansion and differentiation (P. Creemers *et al.*, 1983; Y. M. Kong *et al.*, 1986b). Additional evidence supporting the involvement of cytotoxic T cells in the effector phase of EAT comes from the study of Ben-Nun and colleagues who demonstrated in thyroid transplantation experiments, that H-2 restriction of thyroid damage relied on the derivation of the transplanted thyroid (A. Ben-Nun *et al.*, 1980), (see section 1.2.2.1.1). The modulation of disease severity by H-2K and H-2D region products further argues for cytotoxic cell involvement in thyroid damage (see section 1.2.2.1.1). Moreover, examination of the thyroid infiltrate at various time points of EAT development revealed that the early predominance of Lyt-1$^+$ cells was followed by an increase of Lyt-2$^+$ cells resulting in Lyt-1$^+$: Lyt-2$^+$ ratios very different from those in peripheral blood (P. Creemers *et al.*, 1984). The significance of both helper and cytotoxic T cells as effector cells in murine EAT was recently reexamined (J. C. Flynn *et al.*, 1989) using the adoptive transfer of MTg-sensitized cells as a method for EAT induction. The participation of the two T-cell subpopulations of both donor and recipient mice was studied at various time points of disease activity by selective depletion of respective populations with MoAbs. It was demonstrated that L3T4 cells were the important cells for EAT induction, whereas both L3T4 and Lyt-2$^+$ cells had an effect on the severity of the disease. The involvement of Th cells in the

inductive and effector phase of EAT was further supported by the study of Stull and coworkers, (1988). Using the same adoptive transfer system, they were able to prevent or arrest EAT development in recipient mice by treating the donors or the recipients with anti-L3T4 MoAb.

Regulatory cells: Evidence for the existence of regulatory cells that suppress the development of autoimmune thyroiditis comes from both spontaneous and induced animal models. Early studies in both OS chickens (G. Wick *et al.*, 1970) and BUF rats (D. A. Silverman & N. R. Rose, 1974) demonstrated the critical role of thymus-derived cells in thyroid autosensitization. Neonatal thymectomy of the animals increased the incidence and/or severity of thyroid damage in both species. Analogous results were revealed by Penhale and colleagues who developed a model of SAT in rats by neonatal thymectomy and irradiation (W. J. Penhale *et al.*, 1973) without the requirement of immunization with Tg (see section 1.2.1.2). Development of SAT, however, was abrogated in the thymectomized animals after reconstitution with lymphoid cells from syngeneic recipients. This suggests the presence of regulatory cells within the lymphoid cell population (W. J. Penhale *et al.*, 1976). Similarly thymectomy of certain mouse strains within the second to fourth day following birth resulted in SAT (A. Kojima *et al.*, 1976a). The full development of the disorder, however, could be prevented in the thymectomized recipients by adoptive transfers of lymph node and spleen cells from old mice or thymic cells derived from very young syngeneic donors (A. Kojima *et al.*, 1976b). To explain this difference in the effectiveness of thymectomy over time it was hypothesized that regulatory cells appear in the thymus in early life but shortly after birth migrate to the peripheral tissues where they participate in the regulation of autoimmune responses (N. R. Rose *et al.*, 1981). The presence of those regulatory cells in the periphery of adult animals was demonstrated by recent studies of Sugihara and

colleagues. They induced thyroiditis in mice after depleting them of particular T-cell subsets. The regulatory T-cell subset was shown to express the Lyt-1^{bright} phenotype (S. Sugihara *et al.*, 1988; S. Sugihara *et al.*, 1990).

Kong and coworkers, (1982) were able to induce artificial suppression in EAT susceptible mice after manipulation of the Tg circulatory levels. They treated mice either with soluble deaggregated Tg or with TSH (M. Lewis *et al.*, 1987). Both treatments increased the circulatory Tg levels but via different pathways (exogenous or endogenous). Immunological tolerance was expressed as reduced antibody titers, reduced *in vitro* lymphocyte proliferation to mouse Tg and a low incidence of thyroid lesions. The suppressive effect in both cases was attributed to an antigen-specific T-cell subset bearing the $CD4^+CD8^-$ phenotype and was shown to operate at the afferent phase of EAT (Y. M. Kong *et al.*, 1989; B. E. Fuller *et al.*, 1993). The suppressive effect of both the above treatments was long lasting, but differed in duration depending on the treatment employed to induce that suppression. For example, in animals treated with soluble deaggregated Tg the suppression was detectable for at least 73 days post-treatment whereas in animals treated with TSH it was detectable for only 66 days (B. E. Fuller *et al.*, 1993). On the basis of these studies it was hypothesized that suppressor cells exist naturally in normal mice and elevation of the circulating Tg levels above a threshold results in their differentiation and expansion (Y. M. Kong *et al.*, 1989). Rose and colleagues developed an *in vitro* system that permitted the assessment of the suppressor effect of such regulatory cells. The test depended upon the ability of T cells, derived from mice treated with deaggregated Tg, to inhibit autoantibody production by syngeneic B cells in culture (N. R. Rose & E. Taylor, 1991). Regulatory cells were detected in the thymus as well as in the spleen and were characterized phenotypically as $CD4^-CD8^+$. Their existence is genetically controlled since absence of those cells was

demonstrated in particular strains e.g. SJL. Their inhibitory effect was prominent not only *in vitro* but also *in vivo* since they were effective in suppressing autoantibody production in SCID mice (N. R. Rose & E. Taylor, 1991).

In summary, T cells play a critical role in both the induction, severity and prevention of EAT. Peptide sequences exist (T-cell determinants or epitopes) that associate with certain H-2 gene products. These peptide-MHC complexes stimulate particular T-cell subsets. The final outcome, "health" or "disease", depends on which peptides are generated during Tg processing and on the functional role of the T cells recognizing such complexes. If the clonal balance between effectors and regulatory cells is disturbed, then autoimmunity is established.

1.2.3.2 B cells

Although the role of T cells in autoimmune thyroiditis is well established the role of B cells and their products is still controversial. The contribution of B cells in the disease process has been clearly addressed in the chicken model of thyroiditis because, in that model, the B-cell effects can be eliminated easily by bursectomy. Following such an approach it was shown that the extent of B-cell influences in thyroiditis depends upon the nature of the animal model to be studied. For instance, in the induced chicken model of thyroiditis, neonatal bursectomy decreases EAT susceptibility only minimally (B. D. Jankovic *et al.*, 1965), whereas in the spontaneous model of OS chicken, it reduces significantly both the frequency and the severity of SAT (G. Wick *et al.*, 1970a).

In mice, the role of B cells in EAT has been studied in both actively immunized animals (L. S. Rayfield *et al.*, 1989; A. O. Vladutiu, 1989) and adoptively transferred recipients with *in vitro* activated MTg-primed spleen cells (H. Braley-Mullen *et al.*,

1994). In both cases, B cells were depleted by treatment of the immunized animals or the adoptively transferred recipients with MoAbs specific for B-cell surface IgG. On the basis of those studies it has been demonstrated that B cells are not essential for disease induction although they have a prominent positive effect on the severity of the disease.

To explain B-cell influences on thyroiditis, two alternative and not mutually exclusive hypotheses have been proposed. First, B cells might trigger or maintain the disease cascade by virtue of their effectiveness in presenting autoantigen to autoreactive T cells at low Tg concentrations (A. K. Abbas *et al.*, 1985). Second, the immunoglobulins secreted by B cells could be pathogenic to the thyroid (see section 1.2.3.3). Evidence supporting the first notion comes from the study of Hutchings and colleagues, (1987). Using an *in vitro* system, they demonstrated that in contrast to other professional APC, Tg-primed B cells could trigger the activation of a Tg-specific T-cell hybridoma at low concentrations of Tg. The *in vivo* relevance of such a system, however, remains undefined.

1.2.3.3 Antibody

Evidence from human studies suggests that both anti-Tg and anti-TPO antibodies constitute markers of thyroid autoimmunity (reviewed in C. L. Burek & H. S. Bresler, 1990). The existence of these antibodies indicates predisposition to autoimmune thyroiditis because disease has not been observed in their absence although their presence is not always associated with a pathologic condition (see section 1.1.2.2).

In animal models of thyroiditis the question of the role of antibody has been addressed either directly by adoptive transfers of serum or indirectly based on

correlations between autoantibody levels and EAT. On the basis of those studies, it has been shown that mild thyroid lesions can be transferred by immune serum derived from animals actively immunized with Tg in CFA in several species such as mice (A. O. Vladutiu & N. R. Rose, 1971b; V. Tomazic & N. R. Rose, 1975), guinea pigs (T. Godal & R. Kåresen, 1967; G. C. Sharp *et al.*, 1967) and rabbits (R. M. Nakamura & W. O. Weigle, 1969; K. Inoue *et al.*, 1993b). In contrast, similar transfers of homologous serum in rats (N. R. Rose *et al.*, 1973) and monkeys (I. M. Roitt & D. Doniach, 1958) have no pathogenic effect in the thyroids of the recipient animals. In some cases the successful initial transfers of the lesions by immune serum have not been reproduced. For example, Sugihara and colleagues, (1988) have failed to induce thyroid lesions by infusing immune serum derived from diseased animals into "B mice"**. Similarly, weekly intravenous injections of serum containing antibodies to Tg or to other thyroid antigens into susceptible ($H\text{-}2^k$) mice have failed to transfer lymphocytic thyroiditis (I. Okayasu, 1985).

Correlations between circulating autoantibody titers and severity of EAT have not been generally observed and whenever they were found, proved to be weak and species-dependent. For example, in chickens (G. Wick *et al.*, 1970a) a good correlation between Tg autoantibody levels and magnitude of thyroid lesions has been observed, but in rabbits (R. M. Nakamura & W. O. Weigle, 1967), guinea pigs (R. S. Metzgar & R. H. Buckley, 1967), mice (A. O. Vladutiu & N. R. Rose, 1972; R. J. Esquivel *et al.*, 1978), rats (H. S. Lillehoj & N. R. Rose, 1982) and humans (reviewed in J. Charreire, 1989) such a correlation has not been found. Thus no conclusion can be drawn with respect to the importance of the antibody response in EAT pathogenesis because such a response

** B mice were generated by adult thymectomy and treatment 2 days later, with anti-thymocyte serum. Three weeks later the mice received total body irradiation (850 R) and immediate reconstitution with syngeneic T-cell depleted bone marrow cells (S. Sugihara et al., 1988).

may involve a wide variety of immunoglobulins in terms of specificity, affinity and function. Thus, only a small portion of anti-Tg antibodies may contribute to tissue injury (pathogenic) whereas the rest may be harmless to the thyroid ("bystander"), (reviewed in A. O. Vladutiu, 1990). To address the humoral contribution in autoimmune thyroiditis it is necessary to generate monoclonal or at least oligoclonal antibodies specific for Tg and subsequently test the ability of these reagents to transfer EAT. Initial attempts to transfer thyroiditis in mice using a mixture of eight different MoAbs representing five classes and subclasses of mouse immunoglobulins did not result in disease (reviewed in A. O. Vladutiu, 1990). In subsequent preliminary studies however, Guarnotta and colleagues succeeded in transferring thyroid lesions to mice by MTg-specific MoAbs of IgM or IgG1 class (G. Guarnotta *et al.*, 1982). However, the last results were not reproducible leaving the question of the humoral factors in EAT induction unresolved.

Based on the uncertainty of the previous studies concerning the role of anti-Tg antibodies in the initiation of thyroid damage and on the well demonstrated ability of T cells to mediate such an effect in various species (see section 1.2.3.1), it is reasonable to conclude that humoral factors may play a secondary role in EAT pathogenesis. In fact, anti-Tg antibodies appear to potentiate thyroid damage in several species. For example, OS chickens of B^4B^4 haplotype that normally develop mild thyroiditis, were severely afflicted by the disease after treatment with serum containing a high titer of anti-Tg antibody (J. Jaroszewski *et al.*, 1978). Similarly, the combined transfer of heterologous Tg-specific antibodies and sensitized lymph node cells to guinea pigs augmented the histological alterations in the thyroids of the recipient animals (G. C. Sharp *et al.*, 1974). Furthermore mice treated from birth with anti-IgM MoAb, a procedure that decreases both the numbers of B lymphocytes and the circulatory levels of their secreted

immunoglobulins, and subsequently immunized with Tg in CFA developed thyroid lesions of reduced severity (A. O. Vladutiu, 1989).

Thyroid autoantibodies might induce tissue injury via two different pathways. First, they may be cytotoxic for the thyroid by virtue of their ability to fix complement and mediate thyroid damage either by immediate lysis of thyroid cells (see next paragraph) or by repeated or continuous sub-lethal complement attacks (A. P. Weetman *et al.*, 1990). Second, they may confer specificity of thyroid damage by activating non-specific, Fc-receptor bearing killer cells (antibody dependent cell cytolysis). In both cases, it is required that the thyroid antibodies bind to the surface of TEC and form immune complexes with surface Tg, TPO or another yet undefined surface thyroid autoantigen. Expression of Tg on the basement membrane of thyrocytes has been shown by *in situ* tissue immunofluorescence in mice injected with rabbit anti-MTg antibodies (reviewed in A. O. Vladutiu, 1990). In contrast, TPO is known to be expressed on the apical surface of thyrocytes which, in an intact thyroid, is not accessible to either antibodies or mononuclear cells (E. L. Khoury *et al.*, 1984; T. Hanafusa *et al.*, 1984). Thyroglobulin-anti-Tg complex depositions have been demonstrated by both immunofluorescence and electron microscopy on the follicular basement membrane in chickens (D. V. Katz *et al.*, 1981), mice (J. A. Clagett *et al.*, 1974) and humans (A. E. Kalderon & H. A. Bogaars, 1977). On the other hand, IgG depositions corresponding to TPO-anti-TPO immune complexes have been rarely detected on the apical surface of thyroid cells of HT glands (E. L. Khoury *et al.*, 1984). This is not surprising if we consider that the follicular cells are polarized and that the penetration of immunoglobulins to the apical surface is obstructed by desmosomes (see section 1.1.1.3).

Complement mediated cytotoxicity. Complement mediated cytotoxicity as a potential pathogenic mechanism in autoimmune thyroiditis has been only partially elucidated.

Conflicting results have been reported in various species suggesting the existence of inter-species variability in the effector mechanisms of thyroiditis. Early studies in mice indicated that the late complement components are not essential for EAT induction. Thus, severe disease has been induced in mice deficient in the fifth complement component (C5-D) by immunization with heterologous Tg in the absence of adjuvant (R. M. Nakamura & W. O. Weigle, 1968). In contrast, following a similar approach in rabbits using animals deficient in the sixth complement component (C6-D), it has been shown that the severity of thyroiditis is considerably reduced (K. Inoue *et al.*, 1993a). Similarly in the rat, complement levels in the serum have been studied throughout the course of disease development (N. R. Rose & Marie-F. Molotchnicoff, 1973). In those animals, a drop in the complement levels correlates well with the severity of thyroid lesions. In humans, Forbes and colleagues, (1962) first observed that serum from HT patients is cytotoxic for autologous thyroid cells in culture (I. J. Forbes *et al.*, 1962). The *in vivo* relevance of such a cytotoxic effect must be of secondary importance in the process of tissue injury because cytotoxic antibodies have been identified both in individuals with overt hypothyroidism and in euthyroid HT patients (L. Chiovato *et al.*, 1993).

Anti-Tg antibodies do not fix complement. This inability is not due to the preponderance of IgG subclasses with non-complement fixation properties (L. C. P. De Carvalho & I. M. Roitt, 1982; A. P. Weetman *et al.*, 1989) but is related to the antigenic features of Tg. It has been suggested that the antigenic sites are not distributed close enough on the Tg molecule to facilitate complement activation (T. R. Adler *et al.*, 1984).

ADCC: Antibody-dependent cell-mediated cytotoxicity has been exclusively associated with microsomal antibodies (E. L. Khoury *et al.*, 1981). As mentioned

previously, Tg-anti-Tg immune complexes have been demonstrated on the basal surface of the thyroid follicular cells in several species. However, inter-species variability has been observed with respect to the ability of such antibodies to activate killer cells bearing Fc receptors. For example, in both guinea pigs and OS chickens, antibodies derived from diseased animals rendered homologous normal lymphocytes cytotoxic for Tg-coated erythrocytes (B. Ringertz *et al.*, 1971; G. Wick *et al.*, 1981). Similarly, serum from HT patients rendered Tg-coated chicken red-blood cells (E. A. Calder *et al.*, 1973), porcine thyroid cells (P. Rodien *et al.*, 1992) or human thyroid cells (U. Bogner *et al.*, 1984) susceptible to lysis by normal human lymphocytes. In the last study, the cytotoxic effect in the serum was attributed exclusively to thyroid microsomal antibodies (U. Bogner *et al.*, 1984) although such a correlation was not observed in other studies (J. Sack *et al.*, 1986; P. Rodien *et al.*, 1992).

1.2.4 Mapping of the pathogenic Tg T-cell epitopes.

1.2.4.1 An overview of the existing knowledge of Tg T-cell epitopes.

Although a vast body of evidence is available to support the crucial role of T cells in both the inductive and the effector stages of Tg-induced EAT, very little is known about the precise mechanisms that generate and maintain it. Progress in that direction has been hindered by both the antigenic complexity and distribution of Tg. As a huge macromolecule, Tg is likely to encompass several antigenic sequences (epitopes or T-cell determinants) recognized by diverse T-cell populations which may function synergistically or antagonistically. Moreover, Tg circulates at low levels in the bloodstream (G. Torrigiani *et al.*, 1969), and influences the natural tolerogenic

mechanisms established to abrogate autoreactivity such as suppression (see section 1.2.3.1) or inhibition via the idiotypic network (M. Zanetti & P. E. Bigazzi, 1981; C. Roubaty *et al.*, 1990; B. Texier *et al.*, 1992a). In the process of elucidation of the cellular and molecular events leading to EAT, it was evident that a simplified system was necessary in which the immunoregulatory effects (synergism or antagonism) of diverse T-cell subsets would be eliminated, so that disease development by a pathogenic Tg T-cell subset could be easily followed. Therefore, interest was focused on the mapping of pathogenic T-cell sequences of Tg.

Despite extensive studies in the past decade, very limited information exists regarding the number and nature of pathogenic T-cell epitopes. This is basically due to the fact that the a.a. sequences of MTg are still undefined and the sequence of human, bovine and part of rat Tg (RTg) only recently have become available (see section 1.1.3.1). In addition, Tg is a large and complex molecule and these features make the characterization of its antigenic fragments by biochemical methods a long and laborious process. Despite its huge size (see section 1.1.3.1) however, Tg is believed to include only a small number of pathogenic T-cell epitopes. As a consequence of the MHC-control of susceptibility to EAT, one would expect that only a few Tg peptides that bind to the thyroiditis-associated allele and form MHC-peptide complexes that are recognized by "self" T cells will be pathogenic (reviewed in C. A. Janeway, Jr., 1994).

In the process of mapping thyroiditogenic T-cell determinants of Tg, several questions have been raised regarding their nature. Are they evolutionarily conserved sequences? Do they differ in terms of antigenicity in high and low responder mice? What is the nature of those epitopes with respect to antigenicity?

Evidence concerning the first question was presented by Kong and colleagues in the beginning of the last decade and was subsequently confirmed by other investigators.

Early *in vitro* assays revealed that proliferative T cells having the potential to transfer thyroiditis to normal syngeneic recipients or to display cytotoxicity for thyroid monolayers recognize shared determinants on HTg and MTg (Y. M. Kong *et al.*, 1986b; L. L. Simon *et al.*, 1986). As with HTg, porcine Tg (PTg) and bovine Tg (BTg) could also activate MTg-sensitized cells to transfer EAT (reviewed in R. C. Kuppers *et al.*, 1988). In analogous studies, MTg T-cell lines, clones (C. G. Romball & W. O. Weigle, 1987) and hybridomas were shown to recognize epitopes on Tg that were highly conserved throughout a large number of species (B. R. Champion *et al.*, 1987b). Such MTg-sensitized clonal populations could transfer thyroiditis to normal syngeneic recipients after *in vitro* activation with MTg or BTg (R. Maron *et al.*, 1983). On the basis of these findings, it was proposed that pathogenic T cells recognize epitopes that are evolutionarily conserved among different species.

Other studies were focused on the nature of Tg T-cell determinants recognized by high and low responders. Both susceptible and resistant mice were primed with MTg or HTg and the proliferative responses of their sensitized lymphocytes were tested *in vitro* to both homologous (MTg) and heterologous (HTg) Tg (L. L. Simon *et al.*, 1985; Y. M. Kong, 1986b). Proliferative T cells from high responders appeared to recognize both species-specific determinants and determinants shared between HTg and MTg. Proliferative cells from low responders were directed only to species-specific determinants. This latter observation suggested that T-cell clones recognizing shared Tg determinants were absent from low responders. This hypothesis was not confirmed by a recent study however, in which EAT was transferred to normal syngeneic recipients by a T-cell line derived from a low responder ($H\text{-}2^b$) strain (R. Zerubavel-Weiss *et al.*, 1992). The pathogenic T cells identified in this study were specific for an epitope cross-reactive to both MTg and BTg (R. Zerubavel-Weiss *et al.*, 1992) and had been generated after

priming of H-2^b mice with BTg and subsequent *in vitro* expansion of the sensitized T-cell clones with MTg. In addition, EAT has been recently transferred to low responders by a $CD4^+$ T-cell clone derived from H-$2^{k/b}$ mice, by immunization with MTg in LPS and activated *in vitro* with either MTg or BTg (Y. Hiyama *et al.*, 1993). The pathogenic clone is H-2^b-restricted and responds in *in vitro* proliferative assays to both PTg and RTg. On the basis of the above findings it is clear that evolutionarily conserved T-cell epitopes of Tg can be antigenic in both high and low responders. T cells recognizing such epitopes are harmless to the thyroid of low responders either because the frequencies of their precursors are low or because they are successfully suppressed by regulatory systems (R. Zerubavel-Weiss *et al.*, 1992) such as suppressor cells or anti-idiotypic antibodies reactive with the TCR of the thyroiditogenic clone (B. Texier *et al.*, 1992a).

T-cell determinants have been classified as immunodominant, subdominant or cryptic (reviewed in G. Gammon *et al.*, 1987). A feature considered during the classification of a certain epitope into one of these categories is its ability to stimulate *in vitro* proliferation of T cells sensitized *in vivo* by the native protein. An immunodominant determinant strongly stimulates the *in vitro* proliferation of such cells whereas a cryptic determinant fails to do so. Both immunodominant and cryptic determinants can successfully stimulate T cells derived from mice that have been immunized with the respective determinant itself in adjuvant. Cryptic T-cell determinants are further classified as "absolute" or "latent" epitopes. The term "absolute" refers to a cryptic determinant whose *in vivo* administration generates T cells responsive to the epitope itself but not to the native protein. Conversely, if the generated T cells respond to both the epitope itself and to high doses of the native antigen, the cryptic determinant is designated as "latent" (reviewed in E. E. Sercarz *et al.*, 1993). Between

the cryptic and immunodominant determinants an additional "subdominant" category exists. A subdominant determinant is one that induces proliferative cells responsive *in vitro* to both the determinant itself and to the native antigen (reviewed in E. E. Sercarz *et al.*, 1993). T cells induced by immunization with the native antigen respond inconsistently *in vitro* to the subdominant determinant (reviewed in G. Gammon *et al.*, 1987).

In the thyroiditis field, the significance of immunodominant and cryptic Tg determinants in the disease process is unknown, because thyroiditogenic T-cell epitopes on Tg are mostly undefined. Thus, in the last three years, interest has been focussed on the mapping of such pathogenic T-cell epitopes of Tg based on two independent strategies. The first strategy is based on the generation of MTg-specific T-cell hybridoma clones and subsequent use of those clones as tools for screening for Tg T-cell epitopes with thyroiditogenic potential. The second strategy uses computerized algorithms to predict potential T-cell epitopes within the Tg sequence and the subsequent testing of the candidate sequences for thyroiditogenicity in animals. The latter approach has been previously used successfully for identification of T-cell determinants in other autoimmune models such as that of experimental autoimmune uveoretinitis (EAU), (T. M. Redmond *et al.*, 1989). However, the same approach had not been employed in EAT prior to this study, possibly because Tg is a huge molecule (1.1.3.1) and the use of a single algorithm for identification of potential T-cell epitopes preselects a relatively large number of candidate sequences.

Following the first approach, two Tg T-cell epitopes have been recently defined. The first epitope is a 9-mer peptide corresponding to amino acid 2551-2559 of HTg and containing T4 at position 2553 (B. R. Champion *et al.*, 1991). Its identification was based on the use of CH9 and ADA2 hybridoma clones. Slight variability in the

responsiveness of the two hybridomas to Tg from various species suggested that the Tg epitopes recognized by them are similar but not identical. Moreover it was shown that both hybridomas responded to the Tg fraction carrying T4 and that their activation was highly dependent on the iodination level of that fraction, suggesting that the pathogenic T-cell epitope was localized at a hormonogenic region of Tg (reviewed in K. Dawe *et al.*, 1993). Since there are only four hormonogenic sites on Tg positioned at residues 5, 2553, 2567, and 2746 (Y. Malthièry & S. Lissitzky, 1987), an array of 5 to 12-mer overlapping synthetic peptides covering the four hormonogenic sites was synthesized and each peptide was tested for its ability to activate the two hybridoma clones (B. R. Champion *et al.*, 1991). In this way, the minimal T cell epitope was mapped to the C-terminal of Tg including the residue 2553 (B. R. Champion *et al.*, 1991). Although the minimal sequence recognized by the hybridoma clones, which corresponds to a 9-mer peptide, was ineffective in inducing thyroiditis after direct challenge, it generated effector cells that, after *in vitro* activation, could successfully transfer thyroiditis to normal syngeneic recipients (P. R. Hutchings *et al.*, 1992). The second Tg T-cell epitope (F40D) defined by the same strategy is a 40-amino acid sequence localized between residues 1672 and 1711 of HTg (B. Texier *et al.*, 1992b). This pathogenic sequence constitutes a part of the <10 kDa porcine Tg tryptic digest fraction that was shown to be thyroiditogenic in CBA mice (J. Salamero *et al.*, 1987). With the aid of a cytotoxic hybridoma HTC2 generated by immunization of mice with PTg, it was shown that syngeneic macrophages, pulsed with porcine Tg tryptic fragments containing the pathogenic epitope, were lysed in a dose-dependent manner (reviewed in C. Bedin *et al.*, 1992). To identify within the 10 kDa fraction of PTg the sequence(s) responsible for the activation of HTC2 cells the <10 kDa fraction of PTg was separated by 2D-gel electrophoresis. Those products of electrophoresis that could successfully activate the

HTC2 cells were collected and further purified by high-performance liquid chromatography (HPLC). HPLC fractions corresponding to the major peak that could successfully activate the HTC2 cells were used for N-terminal sequencing. Following the above procedure the highly hydrophobic sequence F40D was defined within the 10 kDa fraction of PTg, that could transfer minimal thyroiditis to CBA mice after direct subcutaneous challenge (B. Texier *et al.*, 1992b).

From the previous discussion it is evident that mapping of T-cell epitopes with the aid of Tg-specific T-cell populations is a difficult task. First, the process of generation of T-cell clones is itself long and laborious. Second, the technical procedure that is involved in the separation from among protein digests those fragments that activate such clonal populations and their subsequent sequencing are complex. Third, in the case of immunization with MTg in mice (homologous system) an additional problem exists. From our experience, we found it extremely difficult to establish MTg-specific clonal T-cell populations in mice possibly due to the suppressor mechanisms that operate inhibiting the activation of autoreactive cells. Analogous observations have been reported by other investigators (C. G. Romball & W. O. Weigle, 1984). In their study it is highlighted that the T-cell response to a homologous Tg is markedly deficient compared to the response to heterologous Tg. Finally MoAb induced by intact Tg will only recognize immunodominant epitopes. Hence, cryptic epitopes can be missed by such an approach. Since the individual significance of immunodominant and cryptic T-cell epitopes in the spontaneous disease process is completely unknown, an alternative strategy that can detect both types of determinants was explored. In the current study, we attempted to define pathogenic epitopes of Tg based on the algorithm approach.

1.2.4.2 Algorithm-based prediction of T-cell epitopes.

It has been well established that the binding of T cells to antigen involves a ternary complex of protein antigen, MHC molecule and T-cell receptor. The antigen must be proteolytically processed by an accessory cell such as macrophage, dendritic cell or B cell. The processed antigenic fragments are subsequently presented to T cells on the surface of the accessory cell after association with an MHC molecule (reviewed in R. H. Schwartz, 1985). The binding of the antigenic peptide on the MHC molecule probably stabilizes its conformation and allows its recognition by the TCR leading to activation of the T cell. The antigenic site has been postulated to form an amphipathic helical structure characterized by both hydrophilic (polar) and hydrophobic (apolar) portions (C. DeLisi & J. A. Berzofsky, 1985). It has been proposed that the apolar face of such a conformation stabilizes via hydrophobic forces the interaction with the APC, whereas the polar face confers specificity on the interaction with the TCR (C. DeLisi & J. A. Berzofsky, 1985). Alternatively, because amphipathic helical structures exhibit a natural affinity for lipid membranes, they may selectively accumulate on the surface of APC in numbers sufficient to saturate MHC molecules of low affinity thus increasing the probability of activation of specific TCR (C. DeLisi & J. A. Berzofsky, 1985; reviewed in J. A. Berzofsky, 1988). Amphipathic helicity might also render the structure less susceptible to protein degradation and thus facilitate the escape of the antigenic determinant from fragmentation during antigen processing (reviewed in J. A. Berzofsky, 1988). Analysis of other antigenic properties of peptides recognized by Th cells revealed a tendency to form α-helical conformations (J. L. Spouge *et al.*, 1987). A peptide is characterized as α–amphipathic when it adopts a helical conformation characterized by one hydrophobic and one hydrophilic side. An analysis of 12 antigenic sites in 6

proteins revealed that 10 of them were satisfying the α-helical hypothesis (C. DeLisi & J. A. Berzofsky, 1985). Considering the predisposition of antigenic sites to form amphipathic structures, Margalit and colleagues developed an algorithm that could predict potential T-cell epitopes within protein sequences (H. Margalit *et al.*, 1987). On the basis of this algorithm, a computer program was formulated designated as AMPHI. This can be used to screen for possible antigenic sites in any protein sequence. Such a prediction is based on the following strategy. The amino acid sequence of a certain protein is converted to a sequence of hydrophobicity values. The hydrophobicity sequence is subsequently divided into overlapping blocks. The first block extends from residue 1 to residue l (where l=7 or 11 a.a.), the second from 2 to l+1, etc. Block length l of 7 and 11 a.a. corresponds to two and three turns of an α–helix respectively. Each block is tested for periodicity in hydrophobicity to demonstrate whether or not it can form an amphipathic helical structure. The final step is to distinguish between stable and unstable amphipathic helices. After having predicted all the possible amphipathic helical segments within a protein sequence, it is possible to grade them hierarchically in terms of amphipathicity based on their amphipathic score. The amphipathic score is the sum of amphipathic indices of the blocks that compose a particular segment. Based on the proposed algorithm, 18 out of 23 known immunodominant Th antigenic sites located on 12 different proteins could be predicted ($P<0.001$) i.e. The ability of the AMPHI algorithm to predict precisely the antigenic sites is limited to 75% (H. Margalit *et al.*, 1987). However a number of consideratio .s have to be kept in mind when considering algorithmic analyses. For example, the algorithm may not be recognizing all amphipathic sequences or alternatively it could be producing false positive predictions. Therefore, although based on this algorithm's results one may be tempted to conclude that an antigenic site is not necessarily amphipathic the above possibiliti-s cannot be

excluded. Therefore, once determined, further testing of the candidate peptides is required because additional factors such as the genetic constitution of the animal and the ability of the candidate peptide to be generated by antigen processing, etc., contribute to its antigenicity (reviewed in J. A. Berzofsky, 1988).

In the process of searching for antigenic sites, Rothbard and Taylor analyzed the primary sequences of 57 known helper and cytotoxic T-cell determinants in four different proteins of human, mouse or guinea pig origin in an attempt to detect common motifs. On the basis of this analysis, they formulated two patterns of consecutive residues that could predict eight new helper and four cytotoxic T-cell epitopes (J. B. Rothbard & W. R. Taylor, 1988). Their work stressed the importance of the side chain contacts of an epitope for selective binding to the MHC. The first pattern is a four residue motif composed of a charged residue or glycine, followed by two hydrophobic residues and a polar residue or glycine. The second pattern is a pentamer motif composed of the first three residues of the previous motif, followed by a hydrophobic residue or proline ending with a polar residue or glycine. Within the 57 known T-cell epitopes tested, the tetramer motif was identified in 46 (81%) whereas the pentamer in only 18 (32%). Although no physical interpretation has been given for those motifs it is noteworthy that the tetramer motif corresponds to one turn of an amphipathic a-helix (reviewed in J. A. Berzofsky, 1988).

Of the two algorithms mentioned previously the former characterizes many determinants regardless of their MHC restriction whereas the latter characterizes peptides that bind to a limited number of MHC alleles (reviewed in J. B. Rothbard & M. L. Gefter, 1991). Recent studies however, have focussed on the identification of specific motifs that allow peptides to bind to certain MHC class II alleles. In that regard, structural motifs have been defined for both non-self peptide sequences that stimulate T

cells in the context of MHC class II alleles (J. Leighton *et al.*, 1991; J. I. Krieger *et al.*, 1991) and for self peptide sequences acid-eluted from purified MHC class II molecules (R. M. Chicz *et al.*, 1992; R. M. Chicz *et al.*, 1993; H. Kropshofer *et al.*, 1992; A. Y. Rudensky *et al.*, 1992). On the basis of those studies peptide binding motifs have been reported for I-A^d (A. Sette *et al.*, 1989), I-E^d (A. Sette *et al.*, 1989; J. Leighton *et al.*, 1991), I-E^k (J. Leighton *et al.*, 1991), I-A^s (A. Y. Rudensky *et al.*, 1992), I-E^b (A. Y. Rudensky *et al.*, 1992), I-A^b (A. Y. Rudensky *et al.*, 1992), DR1 (C. M. Hill *et al.*, 1991; H. Kropshofer *et al.*, 1992; D. O'Sullivan *et al.*, 1991; R. M. Chicz *et al.*, 1992), DR2 (D. O'Sullivan *et al.*, 1991; R. M. Chicz *et al.*, 1993), DR3 (J. Sidney *et al.*, 1992; R. M. Chicz *et al.*, 1993), DR4 (J. Hammer *et al.*, 1993; R. M. Chicz *et al.*, 1993; A. Sette *et al.*, 1993), DR7 (D. O'Sullivan *et al.*, 1991; R. M. Chicz *et al.*, 1993; J. I. Krieger *et al.*, 1991) and DR11(J. Hammer *et al.*, 1993; D. O'Sullivan *et al.*, 1991). Several strategies have been employed to identify such allele-specific structural motifs. One common strategy employed by several groups has involved the usage of analogue peptides containing single-residue amino acid substitutions for binding to a particular class II molecule. Such an approach aims to determine the physicochemical characteristics that are required for each residue within a peptide sequence in order to bind to an MHC-allele(J. Sidney *et al.*, 1992; D. O'Sullivan *et al.*, 1991; A. Geluk *et al.*, 1994). A modification of the above approach which allows quantification of peptide binding with the MHC, has been the production of analogues of class II binding peptides, substituted at each position with long chain-biotinylated lysine. Through this approach it is possible to quantitate binding of each biotinylated analogue to class II by using fluorescent avidin and flow-cytometry (C. M. Hill *et al.*, 1991). Alternatively, the characteristics of peptides binding to MHC class II have been defined by analysing the binding of purified class II molecules to peptides displayed on a phage surface (J. Hammer *et al.*, 1992; J. Hammer *et al.*, 1993).

For this purpose M13 peptide libraries have been produced by introducing oligonucleotides that encode peptides to gene III of M13 phages and have been subsequently screened for binding to a particular HLA class II allele. The structural characteristics of peptides capable of binding to a given HLA allele have been revealed after aligning the peptide sequences of those phages that bind to that allele. On the basis of the above studies it has been shown that for a peptide to bind to a particular MHC class II allele, a limited number of residues exist (anchor residues) that are critical for its binding to that allele. The anchor residues' side chains are known to interact with polymorphic pockets formed by the MHC allele (reviewed in C. A. Janeway Jr., 1994).

Since MHC class II allele-specific motifs were not available at the start of this project, we preselected potential T-cell epitopes within the Tg molecule on the basis of the AMPHI (H. Margalit *et al.*, 1987) and the tetramer motif (J. B. Rothbard & W. R. Taylor, 1988) algorithms. The outcome of such an approach will be described in the next chapters.

1.2.4.3 Significance of non-dominant versus immuno-dominant epitopes in organ-specific autoimmunity.

In the past, interest has been focussed on the identification of immunodominant Th cell epitopes of autoantigens involved in organ specific autoimmunity. This is because Th cells play a central role in the induction of such diseases and the majority of the autoimmune response has been directed to Th determinants. Organ specific autoimmunity to immunodominant T-cell epitopes has formed the basis for designing specific therapeutic strategies (see section 1.2.4.4) to prevent or ameliorate autoaggression. Cryptic determinants of autoantigens, on the other hand, have been less

studied because of the notion that those non-dominant epitopes have a relatively insignificant effect on the induction of autoimmunity. This is the first report demonstrating that pathogenic T cells specific for non-dominant determinants exist in EAT-susceptible individuals. Analogous findings have also been obtained in the autoimmune model of EAU (W. J. Lipham *et al.*, 1991). In that study it was speculated that cells specific for non-dominant T-cell epitopes constituted a potential source of "self"-reactive cells in the periphery of naive animals which could be transformed to autoaggressive cells after activation by various elements such as cross-reactive infectious agents, endogenous or exogenous superantigens or altered "self" antigen. The experimental results of the Lipham group, in conjunction with recent reports suggesting that tolerance is not induced to non-dominant T-cell epitopes when these are components of conjugated peptides (F. Ria *et al.*, 1990) or intact proteins (R. Cibotti *et al.*, 1992), requires that the role of cryptic epitopes in autoimmunity (reviewed in E. E. Sercarz *et al.*, 1993) be re-examined.

There is considerable evidence to suggest that both the antigenicity and tolerogenicity of T-cell epitopes are influenced by similar antigen processing and antigenic competition mechanisms and it has been proposed that T cells recognizing non-dominant T-cell epitopes escape tolerance because the latter are not sufficiently presented to reach the tolerogenic level (G. Gammon & E. Sercarz, 1989). Evidence for such a claim comes from the study of Sasamoto *et al.* (1993). In that study, the investigators attempted to tolerize rats by injecting them intravenously with either immunodominant or non-dominant T-cell epitopes derived from interphotoreceptor retinoid-binding protein (IRBP). They subsequently tested the level of tolerance archieved, as expressed by lack of development of EAU, after injection of the respective epitopes in an antigenic form. Following such an approach it has been shown that both immunodominant and non-

dominant T-cell epitopes are capable of inducing tolerance against themselves. In contrast, when intact IRBP was used, tolerance was restricted to the immunodominant epitopes. The lack of tolerance to non-dominant T-cell epitopes was attributed to their insufficient presentation. In addition, using the same autoimmune model, it has been shown that disease mediated by a non-dominant epitope cannot be inhibited by i.v. immunization of the animals with an immunodominant epitope although such an inhibition is observed when disease is induced by intact IRBP (Y. Sasamoto *et al.*, 1992). Finally, evidence coming from the mouse model of experimental autoimmune encephalomyelitis (EAE) suggests that autoaggression evolves over time. In other words, EAE mediated by intact myelin basic protein (MBP) or the immunodominant epitope Ac1-11 can spread both inter-molecularly to other autoantigens and intra-molecularly to other epitopes, including the non-dominant determinants (P. V. Lehmann *et al.*, 1992; P. V. Lehmann *et al.*, 1993). Reports such as these raise concerns regarding the efficiency of specific immunointervention that is based on the immunodominant epitopes of an autoantigen.

1.2.4.4 Therapeutic implications of defined T-cell epitopes in organ-specific autoimmunity.

As mentioned previously immunodominant T-cell epitopes of autoantigens have been used as tools in the process of developing specific immunotherapy. The murine model of EAE constitutes a reference model, in which a variety of specific approaches that aim for the prevention or amelioration of the disease have been employed. Various strategies have targeted each individual element of the trimolecular complex, namely the pathogenic immunodominant epitope, the MHC allele to which that epitope binds

and the TCR recognizing the epitope-MHC complex. One strategy is tolerance induction. Following, for example, tolerance induction with the immunodominant determinant of MBP, Ac1-9, EAE has been inhibited in both neonatal (J. P. Clayton *et al.*, 1989) and adult animals (A. Gaur *et al.*, 1992). Another strategy involves preventing the pathogenic effect of an epitope by blocking the MHC molecule that accommodates that epitope (L. Adorini *et al.*, 1988). For example, based on the sequence of the immunodominant determinant Ac1-9 and taking into account the residues that are critical for T-cell activation and MHC-binding, peptide analogs have been created with high MHC-binding affinity that could successfully reduce the clinical signs of developing EAE (J. L. Urban *et al.*, 1989; D. C. Wraith *et al.*, 1989). The peptide analogs varied from slightly to completely different from the pathogenic epitope and their therapeutic effects have been attributed both to their effectiveness *in vivo* to compete with the pathogenic epitope for binding to the same MHC and to their inability to activate autoreactive T cells. Another approach has focussed on the T-cell subset that recognizes the immunodominant T-cell determinant. This approach requires that the TCR Vβ usage recognizing the determinant-MHC complex is restricted. Evidence of restriction in the TCR V genes has already been shown to exist in PL/J mice, an EAE-susceptible strain. The majority of T cells recognizing the Ac1-9-MHC complex in that strain were shown to employ the Vβ8.2 segment (J. L. Urban *et al.*, 1988). Taking advantage of this restriction of the Vβ usage in encephalitogenic T cells, the onset of EAE has been successfully prevented in PL/J mice by selective depletion of those cells using an anti-Vβ8 specific MoAb (H. Acha-Orbea *et al.*, 1988; J. L. Urban *et al.*, 1988). Finally, a long-lasting therapeutic effect has been achieved by vaccinating susceptible animals with peptide sequences derived from the variable region of the β chain of the TCR and

thus preventing the subsequent development of EAE or even reverting the clinical signs of established disease (A. A. Vandenbark *et al.*, 1989; M. D. Howell *et al.*, 1989).

EAT therapeutic strategies such as these have been hampered by a limited knowledge of pathogenic T-cell epitopes. To employ those approaches, two requirements must be satisfied. First, the number of pathogenic T-cell epitopes must be limited. Second, the TCR usage of Vβ segments must be restricted. If a wide range of epitopes and TCR-Vβ segments are used, strategies such as anti-Vβ antibody therapy and vaccination with TCR Vβ chain peptides cannot be employed, because the deletion or suppression of the large number of T-cell populations that would be required could lead to general immunosuppression of the immunized individuals.

1.2.5 Characterization of the Tg-specific antibody.

In the process of investigating Tg-specific antibodies as risk factors in thyroid autoimmunity interest has been focussed on the characterization of their immunological nature. Based on the fact that such antibodies exist in the serum of both patients and healthy individuals (B. Guilbert *et al.*, 1982; J. Ruf *et al.*, 1985) it has been hypothesized that Tg-specific antibodies might differ between the two, in terms of quality (IgG subclass distribution pattern, affinity) and epitope specificity (M. Bouanani *et al.*, 1989). If this hypothesis is true, differences in the Tg-specific antibodies could be used as diagnostic or prognostic markers to detect thyroiditis in individuals with subclinical hypothyroidism or in siblings of diseased subjects.

In humans, a number of studies have been devoted to the determination of the subclass distribution pattern of the Tg-specific antibody for several reasons. First, a restricted subclass distribution pattern might be associated with an abnormal immune

response to antigenically altered Tg (reviewed in C. L. Burek & H. S. Bresler, 1990). Second, a restricted pattern may indicate whether or not antibody-mediated mechanisms are essential in the disease pathogenicity (see section 1.2.3.3). Finally, such a pattern might provide some evidence regarding the epitope specificity of those antibodies (N. Fukuma *et al.*, 1989). It has been shown that the IgG subclass distribution of Tg-specific antibody is indeed restricted. The relative proportions, however, of individual IgG subclasses vary depending upon the patient sample studied (S. M. McLachlan *et al.*, 1987) and the method employed for IgG subclass determination (A. B. Parkes *et al.*, 1984; A. P. Weetman & S. Cohen, 1986; M. E. Devey *et al.*, 1989; A. P. Weetman *et al.*, 1989). In mice, the Tg-specific IgG response has been compared in both susceptible and non-susceptible strains (L. C. P. De Carvalho & I. M. Roitt, 1982). No detectable differences in the IgG subclass distribution pattern have been revealed that could explain the striking differences in disease susceptibility between high and low responders (L. C. P. De Carvalho & I. M. Roitt, 1982).

Numerous studies have examined the binding specificities of anti-Tg antibodies directed to either homologous (MTg) or heterologous Tg (HTg) (reviewed in R. C. Kuppers *et al.*, 1991). Those studies have been based on two cardinal strategies. The first strategy relies on the production of MoAbs specific for Tg. The fine specificity of those antibodies is unknown but they can be classified into clusters of reactivity based upon their ability to cross-inhibit binding of other MoAbs to Tg. MoAbs of the same cluster are thought to recognize antigenic determinants close to each other on Tg, whereas MoAbs of different clusters recognize Tg determinants distant from each other. Employing cross-inhibition experiments between MoAbs and serum derived from patients or normal individuals, it is possible to identify those clusters of reactivity that are associated with pathogenicity and those that correspond to natural autoreactivity.

Based on this approach, a limited number of antigenic B-cell determinants on both MTg and HTg have been identified which, in contrast to T-cell determinants, are species specific (B. R. Champion *et al.*, 1987b). In fact, four to six determinants have been found on HTg and five to eight on MTg but their precise amino acid sequence and localization on Tg are unknown (reviewed in R. C. Kuppers *et al.*, 1991). Following a modification of the approach described previously, Charreire and colleagues could define a MoAb to Tg (3B8G9) that could bind to the thyroiditogenic 40-mer sequence F40D (reviewed in K. Mignon-Godefroy *et al.*, 1994).

The second strategy attempts to define B-cell epitopes of Tg based on the primary structure of the protein. To this end, human thyroid cDNA libraries were established and subsequently screened for reactivity with heterologous sera containing Tg-specific antibody. Following such an approach, Dong and colleagues, (1989) have demonstrated 10 epitope-bearing sequences of HTg recognized by rabbit polyclonal sera although none of them was recognized by patients' sera. Using the same method, other investigators have identified three immunodominant regions of Tg located at the extremities and the central part of the molecule. In addition, sera derived from HT patients were shown to react with two of those regions (M. Henry *et al.*, 1990; Y. Malthièry *et al.*, 1991; M. Henry *et al.*, 1992).

Although the methods described previously have contributed to the identification of antigenic regions of Tg, they have certain limitations. The MoAb method can be used only for the identification of immunodominant B-cell determinants because the MoAbs have been raised against intact Tg and therefore are directed to such epitopes. Moreover, for those B-cell epitopes detected by MoAbs the amino acid sequence has not been determined. In contrast to the MoAb method, the cDNA method recognizes only linear epitope-bearing sequences. Any determinant which is conformational or dependent on

postranslational modifications of Tg, such as glycosylation or iodination, can be missed. The current study (see Chapter 8) provides an example of a defined, cryptic, linear T-cell epitope of Tg which has not been detected by previous methods.

CHAPTER 2

THESIS PROPOSAL AND RATIONALE

At the start of this project, the T-cell role in EAT pathogenesis was well established but limited information (see section 1.2.4.1) was available regarding the number and nature of the antigenic determinants on Tg recognized by such cells. Instead of generating clonal populations to use them as tools in the identification of Tg T-cell sites (see section 1.2.4.1), we sought to identify T-cell epitopes among Tg sequences that had been predicted as potential T-cell sites through algorithms (see section 1.2.4.2).

Algorithm-based prediction of T-cell epitopes is a simple method and can be employed for the identification of potentially T-cell reactive sequences in any protein molecule whose primary a.a sequence is available. However, employment of such a method does not assure that the identification of T-cell sites within a given molecule will be successful. This is because other factors outside the determinant site which affect its processing and presentation are also critical (reviewed in G. Gammon *et al.*, 1987). In addition, in several cases T-cell epitopes have been defined by the MoAb method that lack MHC-binding motifs (T. Kotani *et al.*, 1992).

In the past, the algorithm approach has been successfully used for the identification of T-cell epitopes within protein molecules derived from various pathogens such as parasites and viruses with the purpose of designing vaccines (reviewed in J. A. Berzofsky *et al.*, 1987; reviewed in J. A. Berzofsky, 1988). The same approach has also been employed in autoimmunity, for the identification of T-cell sites within autoantigens such as the achetylcholine receptor (R. Hohlfeld *et al.*, 1988) and IRBP (T. M. Redmond *et al.*, 1989) which constitute the principal candidate autoantigens in myasthenia gravis and EAU, respectively.

Encouraged by these successes we proceeded to employ the same method for Tg, the principal autoantigen in EAT (reviewed in J. Charreire, 1989) and most likely in human autoimmune thyroiditis (reviewed in R. Volpè, 1990). Because the primary a.a sequence of MTg was unknown, we used the known portion of RTg as a reference molecule to predict such sites (see section 3.1) assuming that the differences in the a.a. sequence between the two molecules would be minimal. Our assumption was based on several observations. First, the Tg molecule is highly conserved among species (reviewed in G. Medeiros-Neto *et al.*, 1993). Second, the rat is close in phylogeny to the mouse implying homologies between the two species. Third, it has been proposed that pathogenic Tg T-cell epitopes are common throughout species (reviewed in R. C. Kuppers *et al.*, 1988; R. Maron *et al.*, 1983; see section 1.2.4.1).

In the current study, Tg sequences identified as potential T-cell determinants employing the AMPHI and " tetramer motif " algorithms (see section 1.2.4.2), were tested in mice for both immunogenicity and pathogenicity. For this purpose various mouse strains have been used, including susceptible (H-$2^{k,s}$) and non-susceptible (H-$2^{b,d}$) haplotypes to EAT. In analogy to immune responses to foreign or other self antigens such as MBP (reviewed in E. Heber-Katz, 1994) it was assumed that mice of different MHC haplotypes would respond to different Tg determinants. In addition, the level of immunogenicity for a given determinant would be expected to vary among the different mouse haplotypes depending upon the ability of that determinant to bind to MHC, its availability after antigen processing, its ability to induce helper rather than suppressor cells etc. (reviewed in L. Adorini *et al.*, 1988).

What is the value of defining pathogenic Tg T-cell determinants ? It was envisaged that identification of pathogenic Tg T-cell epitopes would provide a simplified model compared to that of Tg, for the study of the disease at both the cellular and molecular

levels (see section 1.2.4.1). The knowledge obtained by those studies could further facilitate our understanding of the immunoregulatory mechanisms that operate in human thyroiditis and in autoimmunity generally. As reported previously (see section 1.1.2.2) HT usually often occurs with other autoimmune diseases such as IDDM, autoimmune adrenalitis etc. suggesting that these conditions share similar etiology. Since such diseases affect organs that are less accessible than the thyroid, conclusions revealed by studies in autoimmune thyroiditis would be valuable in helping us understand other organ-specific autoimmune diseases. Based on that information, specific strategies of immunointervention such as anti-Vβ therapy and anti-clonotypic vaccination could be designed (see section 1.2.4.4). Since immunopathogenic Tg determinants are evolutionarily conserved, pathogenic T-cell epitopes identified in the mouse system might also be pathogenic in humans. Thus, an immunological response directed to such epitopes might be a useful marker of thyroid autoimmunity in humans. In view of the above, the specific aims of this study were:

i) To test the algorithm-based preselected sequences (TgP1, TgP2, TgP3) in genetically diverse mice in terms of pathogenicity and antigenicity.

ii) To classify the thyroiditogenic sequences (TgP1, TgP2) with respect to their antigenicity as immunodominant, cryptic or subdominant T-cell epitopes.

iii) To compare the MHC-restriction requirements of pathogenic sequences with the well established I-A region control of Tg-induced EAT.

iv) To test the nature of the MTg-reactive IgG that is induced after immunization with the thyroiditogenic Tg peptide, TgP1.

CHAPTER 3

MATERIALS AND METHODS

3.1 PREDICTION OF T-CELL EPITOPES WITHIN RAT Tg.

Since the mouse Tg sequence is currently undefined and the Tg molecule is fairly conserved among the species (see section 1.1.3.1), we based our searching for potential T-cell sites on RTg which is phylogenetically close to the mouse molecule. The known portion of RTg sequence, composed of 967 amino acids at the COOH terminal of the molecule (R. Di Lauro et al., 1985), was screened by the AMPHI (H. Margalit et al., 1987) and tetramer motif (J. B. Rothbard & W. R. Taylor, 1988) algorithms. The outcome of such screening by either algorithm is shown in the Appendices 1 and 2. Data presented in Appendix 1 were obtained by the MacIntosh version of the AMPHI program that was kindly provided to Dr. G. Carayanniotis by Dr. J. Berzofsky. Because the number of potential T-cell epitopes predicted by each algorithm was large, it was decided to limit the list by selecting only those sequences predicted by both algorithms. The selected sequences were subsequently listed hierarchically based on the value of their amphipathic score. In table 3.1 the first five sequences characterized by the highest amphipathic scores are shown. The sequences of table 3.1 have been provided by Dr. G. Carayanniotis. From those, three peptides (TgP1, TgP2, TgP3), characterized by high amphipathicity and encompassing one or more tetramer motifs, were further tested for both immunogenicity and pathogenicity in mice (see following chapters) (Table 3.1).

Table 3.1: Prediction of T-cell epitopes on RTg

Amphipathic score	Midpoint of amphipathic segment	SEQUENCE	
67.4	404-432	DSFGQLQGGSQVVKVGTAWKQVYQFLGVP	
39.6	918-934	ADCSFWSKYIQTLKDADGAKDAQLTKS	*
38.8	770-783	SLEHSTDDYASFSRALENATRDYF	**
29.2	49-61	PEGADMATELFSPVDITQVIVNT	
28.1	856-867	LSLKVMQYFSNFIRSGNPNYPH	
25.6	720-729	GLINRAKAVKQFEESQGRTN	***

* TgP2, ** TgP3, *** TgP1 sequences are defined by the vertical lines, ▼ *amphipathic segments* according to the AMPHI program (H. Margalit et al.,1987). *Underlined*, tetramer motif (J. B. Rothbard & W. R. Taylor. 1988).

3.2 ANIMALS AND ANTIGENS

Female C57BL/10J, SJL/J, C3H/HeJ, BALB/cJ, B10.BR/SgSnJ, B10.A, B10.A(2R), B10.A(4R), B10.A(5R) mice were purchased from the Jackson Laboratories, Bar Harbor, ME and were used in experiments at 6-10 weeks of age.

Frozen thyroids of outbred ICR mice and Sprague-Dawley rats (Bioproducts for Science, Indianapolis, IN) were used for the purification of MTg and RTg respectively (see section 3.2.1). HTg was similarly purified from frozen human thyroids. BTg and PTg and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO). Tuberculin purified protein derivative (PPD, Statens Seruminstitut, Denmark) was purchased from Cedarlane, Ontario, Canada. TgP1 (acetyl-GLINRAKAVKQFEESQG-amide), C-TgP1-Y, TgP2 (acetyl-C(acetamide)SFWSKYIQTLKDADGAK-amide) and C-TgP3 (acetyl-C-TDDYASFSRALENATRDY-amide) were synthesized at >70% purity at the Alberta Peptide Institute on an Applied Biosystems (Foster City, CA, USA) 430A synthesizer using a general procedure for solid phase synthesis outlined by Erickson and Merrifield, (1976) with modifications by Hodges et al., (1988). Briefly, hydrogen fluoride cleavage of peptide resin was performed by stirring peptide resin at -5 °C for 1 hr in HF: anisole: dimethylsulfide: p-thiocresol:peptide resin (10 ml:1 ml: 0.5 ml:0.2 ml: 1 g). Peptide purity was determined by HPLC and a.a. analysis. All *in vivo* and most of the *in vitro* experiments utilized TgP1 carrying an N-terminal cysteine and a C-terminal tyrosine that were added to the sequence for cross-linking and labelling purposes respectively. To exclude any possible effect on the antigenic potential of TgP1 created by the presence of the two external to its sequence amino acids, we tested the antigenicity at the B and T-cell level of TgP1 lacking those amino acids (see Chapter 8). Similarly, an N-terminal cysteine was added to TgP3 for cross-linking purposes.

3.2.1 Thyroglobulin purification

For MTg or RTg purification, frozen thyroids of outbred ICR mice and rats were homogenized in phosphate buffer, pH 7.0 and the supernatant was centrifuged three times at 16,000xg. Mouse Tg or RTg was obtained from the supernatant by gel filtration using Sepharose CL-4B (Pharmacia, Baie d'Urfè, Quebec, Canada). By this method, two fractions of Tg were obtained designated as peak I and peak II (see Fig. 3.1). All the *in vivo* and *in vitro* studies were carried out using lyophilized material of the peak II fraction of Tg, which corresponds to fractions 46-59 (fraction size: 80 drops). Peak II was shown to overlap with the eluate of BTg (Sigma, St. Louis, MO) that had been used as molecular marker to standardize the Sepharose CL-4B column.

3.2.2 Heat denaturation of Tg

Heat-denatured Tg was produced according to a modified method of Shimojo et al., (1988) by boiling an aqueous Tg solution (1mg/ml) for 30 min.

3.2.3 Purification of MoAbs

Hybridomas secreting MoAbs specific for I-A^k [k, r, f, or s] (IgG2a) (V. T. Oi et al., 1978), I-E^k (IgG2a) (K. Ozato et al., 1980), Thy 1.2 (J. A. Ledbetter & L. A. Herzenberg, 1979) and NP (influenza A nucleoprotein) (IgG2a) (J. W. Yewdell et al., 1981) were purchased from ATCC (Rockville, MD) and antibodies were purified on protein G-Sepharose affinity chromatography columns (Pharmacia, Baie d'Urfè, Quebec, Canada). The columns were equilibrated with Tris-buffered saline, pH 8.6. The

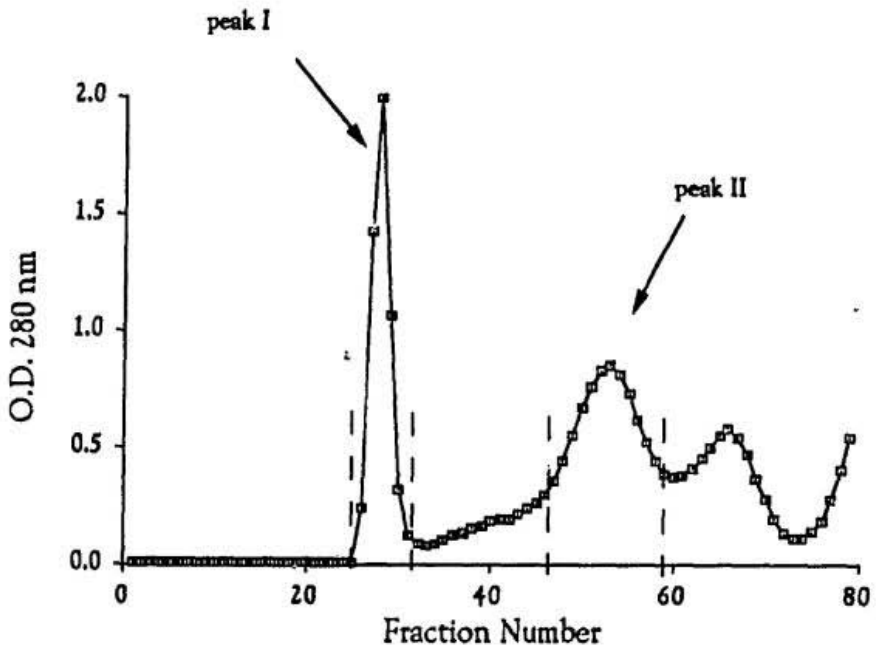


Figure 3.1: Elution profile of RTg from Sepharose CL-4B column. RTg was purified from rat thyroid gland supernatants by gel filtration as described in *Materials and Methods*. Peak II fraction was shown to overlap with the eluate of commercially purified BTg obtained from Sigma.

hybridoma supernatant was subsequently loaded to the column and the antibody was eluted from the column using buffered saline at pH 7.2, 3.5 and 2.3. The fractions corresponding to the elution volume of the antibody were dialyzed against double distilled water (ddH2O) and were subsequently lyophilized and stored at -20.

3.3 EAT INDUCTION

3.3.1 Immunization

Mice were challenged subcutaneously (s.c.) at the base of the tail with 100 nmol of Tg peptide (TgP1=218 µg, TgP2=206 µg, TgP3=224 µg) or 0.15 nmol (100µg) of MTg in 100 µl of 1 : 1 PBS/CFA (with *Mycobacterium butyricum*, Difco Laboratories, Detroit, MI) emulsion. Three weeks later, they were boosted s.c. with 50 nmol of peptide or 0.075 nmol (50µg) of MTg in IFA. Two weeks after the second challenge mice were bled from the retrobulbar sinus to obtain sera for ELISA assays and the thyroids were removed and fixed in buffered formalin.

3.3.2 Adoptive transfer.

Adoptive transfers were performed according to a modification of the protocol of Hutchings et al. (1992). SJL donor mice were immunized with 50 nmol of TgP2 in 100 µl of CFA emulsion or 100 µl of CFA emulsion alone (controls). Ten days after challenge, peptide-primed inguinal LNC were cultured in the presence of TgP2 (10µg/ml) whereas CFA-primed cells were incubated with con A (4 µg/ml). Three days later, the cells were washed 3x with balanced salt solution (BSS) and transferred

intraperitoneally (i.p.) in BSS, to normal syngeneic recipients at $2x10^7$ cells/ animal. Control animals were injected with CFA-primed cells activated *in vitro* with con A. Fourteen days after transfer, recipient animals were bled to obtain sera and sacrificed. Their thyroid glands were collected in buffered formalin, processed, histologically examined and scored as described in the following paragraph.

3.3.3 Evaluation of thyroid pathology

Fixed thyroid-glands were sectioned serially (80-100 sections/mouse). The sections were stained with hematoxylin and eosin and scored* as follows: 0 = no infiltration; 0.5 = interstitial accumulation of inflammatory cells distributed between two or more follicles; 1 = one to two foci of inflammatory cells at least the size of one follicle; 2 = extensive infiltration, 10-40% of total area; 3 = extensive infiltration, 40-80% of total area; 4 = extensive infiltration, >80% of total area.

3.4 CELLULAR ASSAYS.

3.4.1 Proliferation Assays.

Mice were s.c. immunized at the base of the tail with Tg (50 µg) or TgP1 or TgP2 (50-100 nmol) emulsified in CFA. Ten days later, the inguinal lymph nodes were collected aseptically and single cell suspensions were prepared in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Bioproducts for Science, Indianapolis, IN), 20 mM HEPES buffer, 2 mM L-glutamine, 100 U /ml penicillin, 100

* The origin of the tissue was known to the reader.

Chemical Co., St. Louis, Mo.). After centrifugation and washing, cells (4 x 10^5/200 µl/well) were cultured with and without antigen in flat-bottomed 96-well microculture plates and incubated for 4 days at 37 °C in a 5% CO_2, 95% air humidified incubator. At 18 hr before harvesting, 1 µCi of [^{3}H]thymidine ([^{3}H]TdR) (6.7 Ci/mmol, ICN Radiochemicals, Missisauga, Ont.) was added to each well in 25 µl of medium. The cells were harvested using a semiautomatic cell harvester (Skatron Inc. Sterling, VA) and counted in a liquid scintillation counter (LS3801, Beckman Instruments Inc.). Stimulation index (SI) is defined as (cpm in the presence of antigen / cpm in the absence of antigen).

3.4.2 T-cell depletion

LNC ($1.2x10^7$/tube) were suspended in RPMI 1640 in the absence of serum. After centrifugation and washing (2x), the cells were resuspended in 1 ml neat culture supernatant containing Thy 1.2-specific antibody. The cells were incubated with the supernatant for 30 min at room temperature. They were washed 2x in medium without FBS and incubated with rabbit complement (1:10), (GIBCO) which had been previously absorbed with mouse tissue including lymph nodes, spleen and thymus (see section 3.5.4). After 60 min of incubation at 20 °C the cells were washed 2x with medium without serum and resuspended in complete medium. LNC depleted in T cells following this method were subsequently tested for proliferation to TgP1 or TgP2. Controls included cells incubated in the presence of complement but no Thy 1.2-specific MoAb and cells incubated with Thy-1.2-specific MoAb in the absence of complement.

3.4.3 Generation of antigen specific T-cell lines

Antigen-specific T-cell lines were generated according to a modification of the protocol of Champion et al. (1985). Mice immunized with 100 nmol of Tg-peptide were sacrificed 8 days later, and their lymph nodes and spleens were collected in complete medium (see section 3.4.1). The cells were washed twice and placed in flasks at 10^7cells/ml in the presence of 5% con A supernatant* and Tg-peptide (40µg/ml). The cells were incubated for 11 days at 37 °C in a 5% CO_2, 95% air-humidified incubator in 25 cm^2 flasks standing in an upright position. By the end of the incubation period, the viable cells were separated over Ficoll-Hypaque (see section 3.4.4) (Pharmacia, Piscataway, New Jersey) and were restimulated with feeders and antigen for 13 days. The cells were subsequently tested for specificity in a proliferative assay.

Restimulation of the cells: $2x10^6$ viable T cells were placed in 25 cm^2 flasks with $2x10^7$ mitomycin C- treated spleen cells (see section 3.7.1) and peptide (40µg/ml).

Test of specificity: T cells ($2x10^5$/ml) were placed in flat-bottomed 96-well microculture plates with mitomycin C-treated spleen cells ($2x10^7$ /ml) and titrated-concentrations of Tg-peptide (20µg/ml starting antigen concentration). The cells were incubated for 4 days at 37 °C and their proliferative ability was determined via the [^{3}H]TdR uptake method (see section 3.4.1).

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* Supernatant was derived from rat spleen cells cultured *in vitro* with 2-4 µg/ml con A for 3 days. Any additional con A in the supernatant was inactivated by addition of α–methylmannoside prior to use.

3.4.4 Separation of viable cells by Ficoll-Hypaque

The lymphocyte suspension was centrifuged at 300g (1200 rpm) and the obtained pellet was resuspended in BSS. The cells were spun again under the same conditions and resuspended in 4 ml of BSS. They were subsequently layered carefully over 3 ml of Ficoll-Hypaque and were centrifuged at 400g (1400rpm) for 30 min. The interface layer was collected and was suspended in 5 volumes of BSS. The cells were centrifuged at 200g (1200 rpm) for 10 min. The last step was repeated twice and the cells obtained were resuspended in complete medium. Viability of the cells was assessed by the Trypan blue exclusion test. Briefly, cells were resuspended in trypan blue saline solution and loaded to a haemocytometer. The numbers of unstained (viable) white blood cells and stained (dead) cells were counted separately and the percentage of viable cells was calculated as follows:

$$\% \text{ viable cells} = \frac{\text{number of viable cells}}{\text{number of viable cells} + \text{number of dead cells}} \times 100\ \%$$

For greater accuracy in the estimation of viability the total cell number counted was not less than 200 cells.

3.5 DETERMINATION OF ANTIGEN-SPECIFIC IgG RESPONSE

3.5.1 Enzyme-linked immunosorbent assay (ELISA).

Wells of polyvinyl chloride microtiter plates (Dynatech Laboratories, VA) were coated overnight at 4 °C with 0.2 µg of Tg-peptide or 1 µg of Tg in 100 µl of carbonate buffer pH 9.6. After washing, the wells were blocked overnight with 0.1% BSA in PBS. The serum samples were incubated for 1 hr with the antigen and after washing 3x with

200 µl PBS-Tween (PBST), 100 ml of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co.) was added to each well for 1 hr. The plates were washed 3x with PBS-Tween and p-nitrophenyl phosphate substrate was added (1 mg/ml in 10% diethanolamine, 100 µl /well, 1 hr). Absorbance of the p-nitrophenolate product at 405 nm was measured using a Titertek Plus reader.

3.5.2 Competition by TgP1 of TgP1-specific IgG binding to MTg.

Plates were coated with Tg and blocked with 1% BSA in PBS for 2hr. After washing with 0.1% BSA in PBS, they were incubated with both immune serum (50 µl/well) derived from TgP1-primed animals (1:200) and titrated amounts of TgP1 or TgP2 (50 µl/well) for 1 hr. The starting inhibitor concentration was 1.983 µM which corresponds to 4.33 µg/ml of TgP1 or 4.08 µg/ml of TgP2. The following steps were the same as described in the previous section.

3.5.3 IgG- subclass determination

Establishment of standard curves for quantitation of individual IgG subclasses within the serum derived from normal animals (see Fig 3.2) was performed by coating wells with a goat anti-mouse IgG (Fab-specific, 5µg/well) (M-6898, Sigma) followed by blocking with 0.1% BSA in PBS. The wells were then incubated with titrated amounts of a mouse immunoglobulin reference serum (ICN Biochemicals, Costa Mesa, CA, cat.no. 64-901) containing known quantities of IgG subclasses. Subsequently, the wells were washed, and 100µl of neat rabbit anti-mouse IgG subclass-specific antibodies were added for 1 hr (Mouse Typer Sub-Isotyping Panel, cat. no 172-2055, Bio-Rad Laboratories,

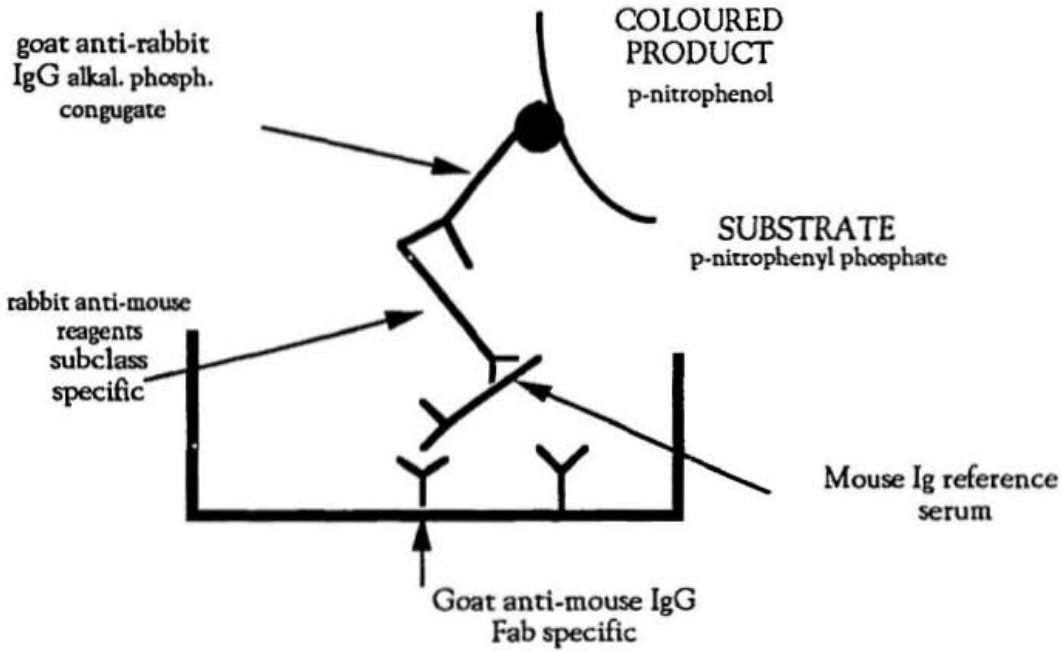


Figure 3.2: Schema describing the procedure followed to make standard curves for individual IgG subclasses.

Richmond, CA) followed by a wash (5x) with PBST. Then the wells were incubated with a goat anti-rabbit IgG (H+L), alkaline phosphatase-conjugated antibody (Bio-Rad) (1:2000) for 1 hr. The data were transformed to linearity using the log form of the von Krogh equation (D. P. Stites et al., 1987) and antibody concentration values in the experimental sera were extrapolated from points in the mid region of the straight lines obtained for each IgG subclass (see Appendix 3). The experimental sera were tested on MTg (1µg/100µl/well) at 1:128 dilution for detection of IgG1 or IgG2b, and 1:32 for detection of IgG2a or IgG3. These dilution points were found in preliminary experiments to yield O.D. values within the appropriate region of the standard curves. The subsequent incubations with rabbit anti-mouse IgG and AP-labelled goat anti-rabbit were as described above.

3.5.4 Absorption of rabbit complement with mouse tissue

Absorption of rabbit complement with mouse tissue was performed according to a modification of a protocol previously described (B. B. Mishell & S. M. Shiigi, 1980). Lyophilized rabbit serum (GIBCO) was reconstituted according to the manufacturer's specifications with 3 ml of distilled water. The spleens and lymph nodes from two animals were removed and a single cell suspension was prepared in BSS. The cells were washed twice and pelletised. Three milliliters of reconstituted rabbit serum were subsequently added to the pellet. The tubes were placed on ice for 30 min. They were then centrifuged for 15 min at 250g (1100 rpm). The supernatant containing the absorbed complement was filtered and aliquoted. Aliquots were kept in -70 ^{0}C.

3.6 TISSUE IMMUNOFLUORESCENCE

Following a previously described protocol (T. I. Michalak et al., 1989), five-micron thick cryostat sections were cut from normal SJL thyroid glands, previously frozen at -80 ^{0}C and embedded in Histo-Prep (Fisher Scientific, Fair Lawn, NJ). The sections were air-dried, fixed in cold acetone for 5 min at room temperature and hydrated in PBS. Susequently, the sections were incubated with TgP1-primed SJL serum at 1:10 dilution for 45 min at room temperature, followed by three washes with PBS. Control sections were similarly incubated with normal SJL serum. Staining was performed with an fluorescent isothiocyanyate (FITC)-conjugated, Fab-specific, goat anti-mouse IgG antibody (Sigma) at 1:120 dilution for 30 min. The sections were again washed 3x with PBS, and were mounted in 20% buffered glycerol prior to being examined for immunofluorescence using a Leitz Diaplan microscope.

3.7 OTHER TECHNIQUES USED.

3.7.1 Treatment of cells with mitomycin C

The spleen-cell suspension was adjusted to 1-6x10^7/ml and incubated with 25 µg of mitomycin C (Sigma, St. Louis, MO) per ml of cell suspension, for 20 min at 37 ^{0} C. Throughout the incubation period the cells were protected from light. After incubation they were washed 3x and used as antigen-presenting cells in the generation of Tg-peptide specific T-cell lines (see section 3.4.3).

3.8 STATISTICAL ANALYSIS

Within each strain, statistical differences between the experimental groups and a control group of seven mice that received adjuvant only and exhibited no infiltration, were determined by the non-parametric Mann-Whitney U test (S. Siegel, 1956).

CHAPTER 4

IDENTIFICATION OF A THYROIDITOGENIC SEQUENCE WITHIN THE THYROGLOBULIN MOLECULE[1]

4.1 SUMMARY

Tg-specific T cells are important in the induction of EAT, but the nature and the number of the Tg T-cell epitopes involved in the disease process are unknown. Through the use of computerized algorithms that search for putative T-cell epitopes, a 17-mer peptide (TgP1) was identified within the known portion of the RTg sequence (corresponding to amino acids 2495 to 2511 of the human Tg sequence) that induced strong mononuclear cell infiltration of the thyroid in classic EAT-susceptible murine strains such as SJL, C3H, and B10.BR and low or undetectable infiltration in EAT-resistant strains such as BALB/c and B10. TgP1 appears to be phylogenetically conserved since it is completely homologous to its bovine counterpart and differs at a single amino-acid position from its human analogue. After priming with TgP1 *in vivo*, significant proliferative T-cell responses to TgP1 *in vitro* were observed only with lymphocytes from susceptible (high responder) strains, thus correlating proliferative capacity with EAT induction. TgP1-primed T cells did not respond to intact MTg or RTg *in vitro* and, conversely, T cells primed *in vivo* with MTg or RTg did not respond to TgP1 in culture, suggesting that TgP1 is composed of non-immunodominant T-cell determinants. TgP1 was defined as a serologically non-immunodominant epitope as well, since *in vivo* priming of all strains with MTg led to strong MTg-specific IgG responses but no TgP1-

[1] Part of the results presented in this chapter has been published as: Chronopoulou E. and Carayanniotis G. 1992. Identification of a thyroiditogenic sequence within the thyroglobulin molecule. *J Immunol.*, Vol. 149: 1039-1044.

specific responses in ELISA assays. This was not due to lack of immunogenic B-cell determinants on TgP1, however, because peptide challenge of EAT-susceptible strains elicited TgP1-specific IgG that also cross-reacted with mouse, rat, human, bovine and porcine Tg. The peak of TgP1 specific antibody response was reached 21 days after priming and was dependent on the peptide dose used for immunization. The data demonstrate that TgP1 delineates non-immunodominant but highly immunogenic determinants at both the T- or B-cell level which may play an important role in the development of autoimmune thyroiditis.

4.2 INTRODUCTION

Murine EAT, an analogue of HT in humans, is an inflammatory autoimmune disease that can be induced by injection of MTg and adjuvant (J. Charreire, 1989). A crucial role for EAT induction has been assigned to T cells since thyroiditis can be adoptively transferred by MTg-specific T cells, $CD4^+$T-cell lines, or clones (H. Braley-Mullen *et al.*, 1985; R. Maron *et al.*, 1983; C. G. Romball & W. O. Weigle, 1987). EAT is genetically controlled by the H-2 complex, and I-A is the main locus regulating EAT susceptibility within the high responder $H\text{-}2^k$ haplotype (A. O. Vladutiu & N. R. Rose, 1971a; K. W. Beisel *et al.*, 1982a). These findings have indicated that MHC-restricted recognition of a limited number of thyroiditogenic MTg peptides by T cells is a key event in the development of the disease. No information exists, however, as to the nature of such peptides, partly because MTg has not been sequenced, and partly because the 660-kDa molecular mass of the MTg homodimer discourages the use of enzymatic techniques that could resolve this issue.

In this study, an attempt was made to identify EAT-inducing epitopes by using a synthetic peptide approach. To stay phylogenetically close to MTg, attention was

focussed on the known portion of the RTg sequence, which consists of the last 967 a.a. at the carboxyl-end of the molecule (R. DiLauro *et al.*, 1985). The sequence was screened by the "AMPHI" and "tetramer motif" algorithms (H. Margalit *et al.*, 1987; J. B. Rothbard & W. R. Taylor, 1988) for potential T-cell epitopes with the view to test candidate peptides, identified by both algorithms, for their ability to induce EAT *in vivo*. Through this approach, we are able to report here the identification of a 17-mer TgP1 that induces EAT, stimulates T-cell responses, and elicits antibodies that cross-react with Tg from various species.

4.3 RESULTS

4.3.1 TgP1 is a conserved epitope and carries multiple MHC-binding motifs.

The rat TgP1 sequence (Fig. 4.1) is identical to its bovine counterpart (a.a. 2497-2513) (L. Mercken *et al.*, 1985) and differs at a single a.a. position from its human analogue (a.a. 2495-2511) (Y. Malthièry & S. Lissitzky, 1987). This is suggestive of a conserved sequence in phylogeny and renders it likely that TgP1 is identical to its murine counterpart as well, constituting a self antigen in the mouse. TgP1 was initially selected as a putative T-cell epitope site because it carries two "tetramer motifs" (J. B. Rothbard & W. R. Taylor, 1988) starting at positions 2495 and 2501 respectively, as well as an amphipathic segment (2500-2509) with a relatively high amphipathic score of 25.6 (block length=11) (H. Margalit *et al.*, 1987). In addition, TgP1 although it is characterized by a lower amphipathic index compared to other sequences (see table 3.1) was selected as a candidate epitope because it lacks internal proline. Presence of proline in the middle of TgP1 would rather favor a turn in the helix thus interrupting its continuity (H. Margalit *et al.*, 1987). TgP1 has structural characteristics of an MHC-binding peptide according to recently described algorithms: it encompasses two

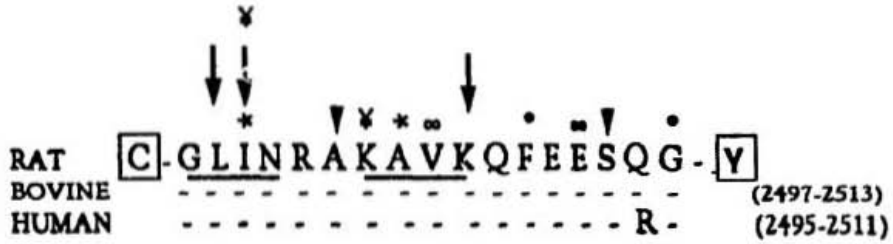


Figure 4.1: Primary amino-acid sequence of TgP1. *Underlines*, Rothbard and Taylor tetramer motifs; *short arrows*, amphipathic segment, according to the "AMPHI" program (amphipathic score=25.6, block length=11); *, ∞ or •, pairs of a.a. obeying the MHC-binding motif of Hill et al., (1991); ¥, a.a delineate an HLA-DR8-binding motif (R. M. Chicz et al., 1993); *long arrows*, a.a. in agreement with the I-E-binding motif of Leighton et al., (1991); -, a.a. identity between rat and bovine or human sequences at the positions shown. All *in vivo* and *in vitro* studies of this chapter utilized TgP1 carrying an N-terminal cystein and C-terminal tyrosine that were added to the sequence for cross-linking and labelling purposes respectively.

Table 4.1: Physicochemical characteristics of TgP1 §

Molecular weight (MW)	1,875
Isoelectric point (pI)	10.08
Half-life *in vitro* mammalian reticulocytes	30 hr
Half-life *in vivo* yeast	>20 hr
Escherichia coli	>10 hr

§ Physicochemical parameters according to the PHYSCHEM program of PCGENE (see section 4.3.1).

sequences with the motif of Hill *et al.* (1991), in which bulky hydrophobic amino acids (Ile2497 and Phe2506) are separated by four residues from a small amino acid (Ala2502 and Gly2511 respectively), and contains an I-E^k-bindin$_g$ motif according to the method of Leighton *et al.* (1991), since two hydrophobic residues (Leu2496 and Ile2497) are separated by six residues from Lys2504 (Fig. 4.1). TgP1 also carries a motif of naturally processed peptides bound to HLA-DR8 (Ile2497-Lys2501), (R. M. Chicz *et al.*, 1993). An analysis of the TgP1 sequence using the PHYSCHEM program of PCGENE revealed the physicochemical features of the sequence which are listed in Table 4.1. The algorithm used in this program to estimate the isoelectric point (pI) starts to calculate the total charge (number of positive-number of negative groups) of a given peptide at pH 7.0. If the total charge of a peptide at pH 7.0 has positive charge this is an indication of existence on that peptide of protons that are not neutralized. Thus the pH value is raised to 10.5 and the total charge is re-estimated. If the total charge at pH 10.5 is negative the pH value is dropped between 10.5 and 7.0 and the total charge is calculated again. This process continues up to that pH value where the total charge will approach 0. That pH value is defined as pI. It should be noted, however, that the pI estimation based on the primary a.a. sequence of a given peptide is only an approximation. This is because the charge of the a.a. side chains of one peptide can be influenced by the environment such as solution conditions, neighbouring a.a. residues in three dimensional space etc. The algorithm that estimates the half-life of a given sequence is based on the "N-end rule" of Bachmair and colleagues (1986). Briefly, the authors have shown that the identity of the N-terminal residue of a protein is critical in determining its stability *in vivo*. It appears that the N-terminal residue is important in the process of ubiquitin-mediated proteolytic degradation. Employing site directed mutagenesis Bachmair and colleagues generated beta-galactosidase proteins with

different N-terminal amino acids. The beta-galactosidase proteins were shown to differ in their half-lives *in vivo*, from more than 100 hr to less than 2 min depending on the amino-terminal a.a. and on the experimental model used (yeast *in vivo*; mammalian reticulocytes *in vitro*; Escherichia coli *in vivo*) (A. Bachmair *et al.*, 1986).

4.3.2 TgP1-induced EAT in mice exhibits a genetic pattern analogous to that obtained after MTg challenge.

In preliminary studies, TgP1 was identified as being able to induce EAT in C3H (H-2^k) mice (Table 4.2). Subsequently, high- and low responder strains were s.c. challenged with TgP1 or MTg to test whether TgP1 mediated EAT was under similar genetic control as EAT induced with intact MTg. The results (Fig. 4.2) demonstrated that TgP1 could induce significant mononuclear infiltration of the thyroid in classic high responder strains such as C3H and B10.BR (H-2^k) or SJL (H-2^s) (A. O. Vladutiu & N. R. Rose, 1971a). In contrast, after TgP1 challenge, no significant infiltration was observed in BALB/c (H-2^d), and no infiltration at all was detected in B10 (H-2^b) mice. Both of these low responder strains exhibited low but significant infiltration indices after challenge with MTg, perhaps due to influences of other thyroiditogenic epitopes on MTg. The dramatic difference in TgP1-induced infiltration between the H-2 congenic strains B10.BR and B10 indicated that this process is under the direct control of genes located within the H-2 complex, and analogous to the findings with native MTg (V. Tomazic *et al.*, 1974).

The EAT lesions induced by TgP1 varied in appearance ranging from small inflammatory foci, especially at low infiltration indices (Fig. 4.3 A, B), to diffuse mononuclear cell infiltrates accompanied by follicular destruction at higher infiltration

TABLE 4.2: EAT induction in C3H mice by TgP1

	Infiltration Index						
Antigen *in vivo*[a]	0	0.5	1	2	3	4	Mice with EAT
TgP1[b]	1	0	1	3	1	0	5/6
CFA	5	0	0	0	0	0	0/6
MTg	0	0	0	1	4	0	5/5

[a] C3H mice were immunized with TgP1, MTg, or PBS in CFA as described in *Materials and Methods*.

[b] TgP1 carried an N-terminal cystein and a C-terminal tyrosine added to the sequence for cross-linking and labeling purposes respectively.

Figure 4.2: EAT induction measured as infiltration index in individual mice from different strains after challenge with TgP1 or MTg. Immunization and histologic assessment of thyroid infiltration were performed as described in *Materials and Methods*. p values were obtained by comparing infiltration in TgP1 or MTg injected mice to infiltration in mice injected with CFA-PBS only (non-parametric Mann-Whitney U test).

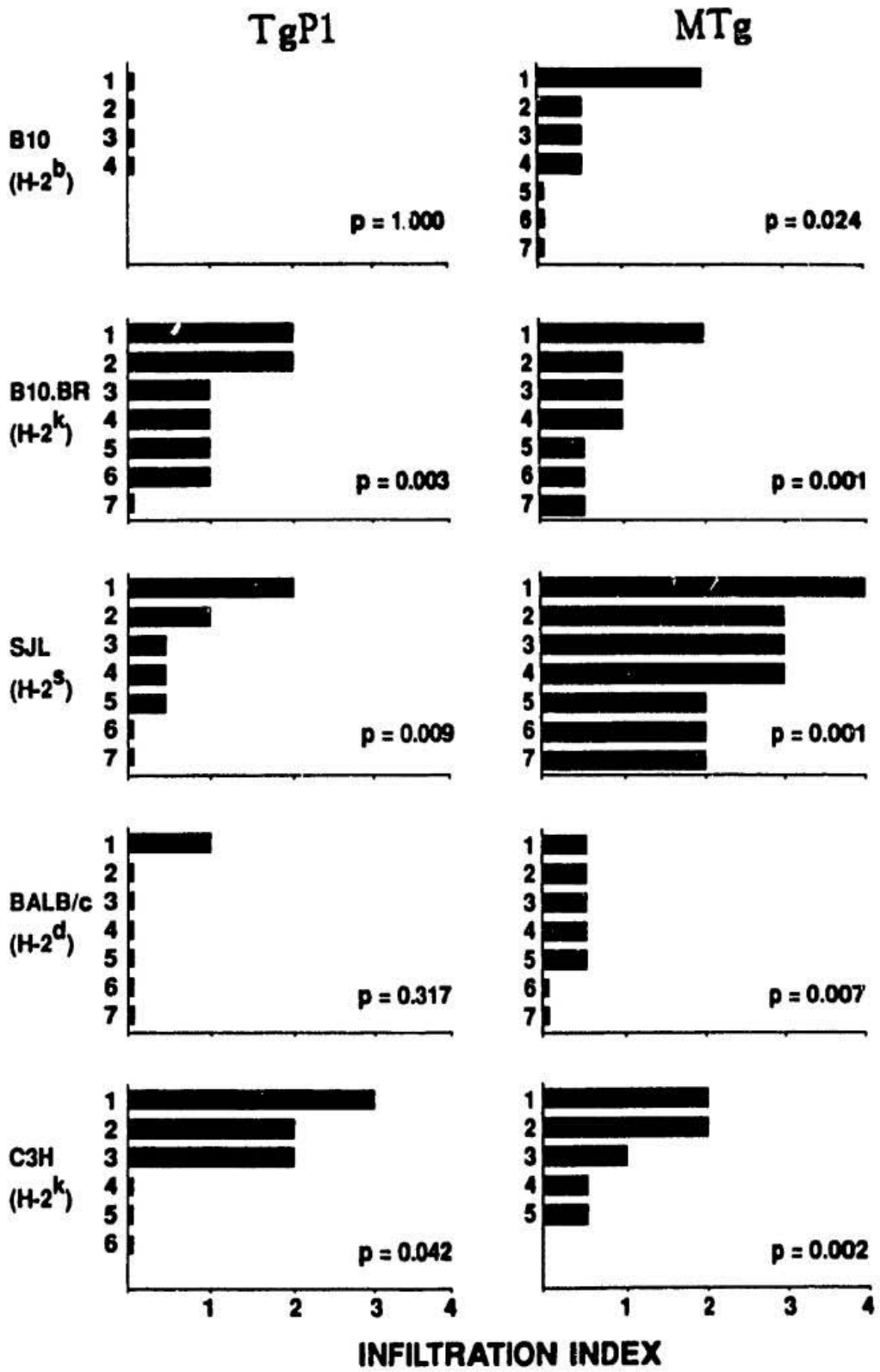
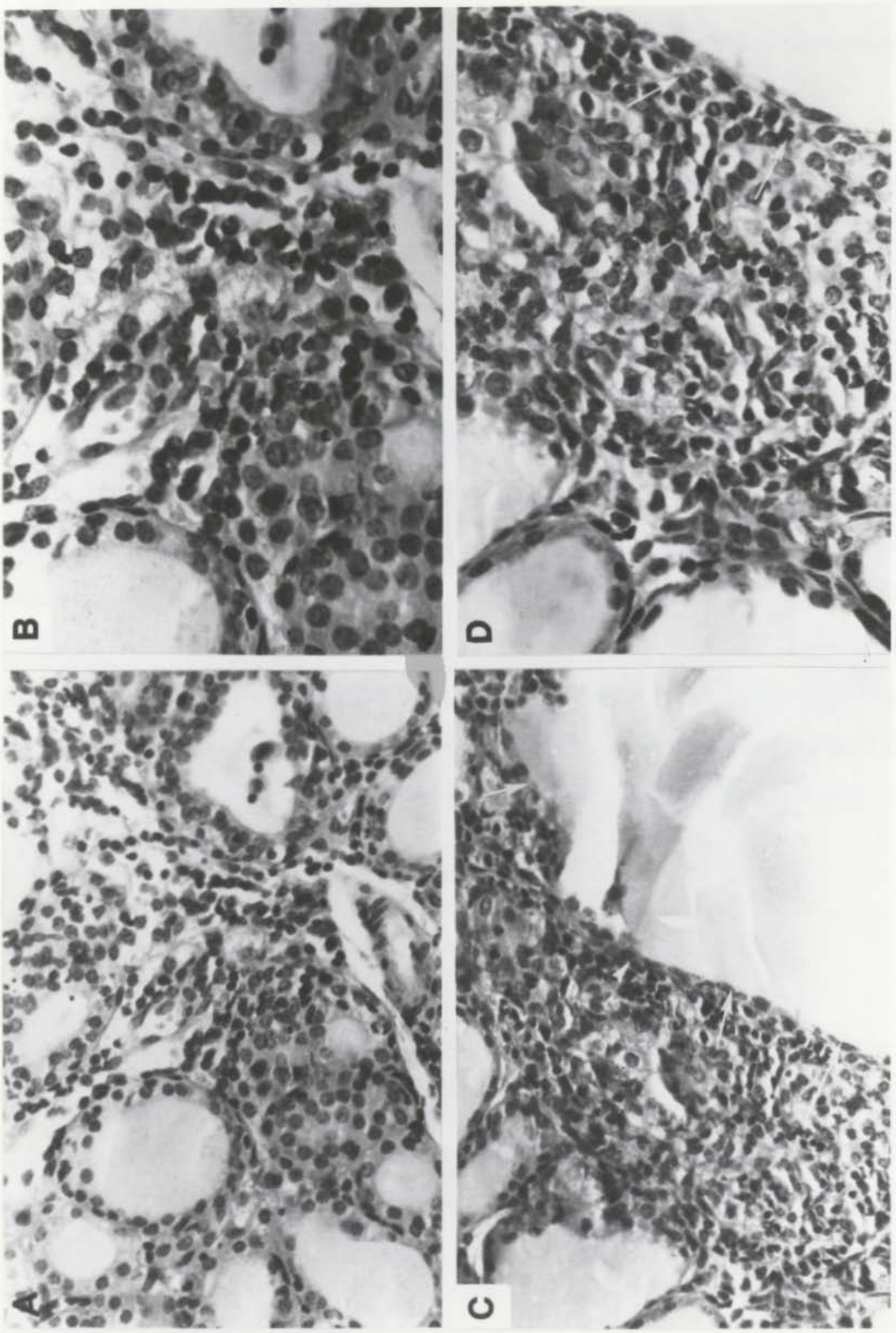

TgP1
MTg
B10
(H-2b)
B10.BR
(H-2k)
SJL
(H-2s)
BALB/c
(H-2d)
C3H
(H-2k)
p = 1.000
p = 0.024
p = 0.003
p = 0.001
p = 0.009
p = 0.001
p = 0.317
p = 0.007
p = 0.042
p = 0.002
1 2 3 4
INFILTRATION INDEX

Figure 4.3 A-D: TgP1-induced interstitial infiltration of mononuclear cells in the thyroid. Thyroid infiltrate ranged from small foci with limited follicular distraction, A (X250), B (X400) to extensive replacement of the thyroid with inflammatory cells C (X250), D (X400). *White arrows* point to a giant follicle(C, D).


B
D
A
C

levels. Occasionally, giant follicles with little colloid and surrounded by inflammatory cells were observed (Fig. 4.3 C, D).

4.3.3 TgP1 elicits specific proliferative T-cell responses that correlate with EAT induction.

To determine the immunogenicity of TgP1, mice were s.c. challenged at the base of the tail with 100 nmol of peptide, and 10 days later the proliferative responses of their inguinal LNC were determined. As shown in Fig. 4.4, vigorous responses were obtained from lymphocytes of SJL, C3H and B10.BR strains that expressed the highest degree of thyroid mononuclear infiltration after TgP1 challenge. BALB/c lymphocytes proliferated less well, and B10 lymphocytes were completely unresponsive to TgP1. LNC proliferation was abrogated after lymphocyte treatment with an anti-Thy1.2 MoAb plus complement (C'), suggesting specific recognition of TgP1 by T cells (Table 4.3). These results confirmed the predictive value of the algorithms used for identifying the TgP1 sequence and revealed complete correlation between T-cell reactivity and thyroiditogenicity of TgP1.

4.3.4 TgP1 does not encompass immunodominant T-cell determinants.

To test whether TgP1 is an immunodominant epitope, we examined the capacity of MTg-sensitized lymphocytes to recognize TgP1 as well as the capacity of TgP1-primed lymphocytes to recognize intact Tg *in vitro*. To exclude that unresponsiveness might be due to lack of TgP1 determinants within MTg, RTg was used in parallel, for *in vivo* priming or *in vitro* testing of lymphocytes. As seen in Table 4.4, MTg-primed

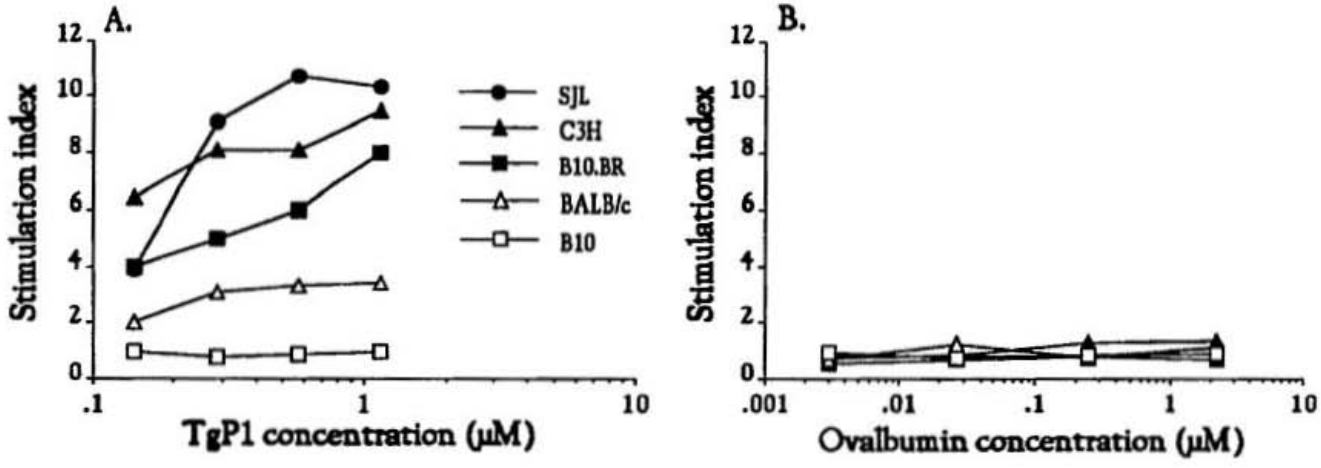


Figure 4.4: Proliferative LNC responses in mice of H-2 haplotypes to TgP1 (A) and OVA (B). Mice were s.c. immunized at the base of the tail with 100 nmol TgP1, and 10 days later the inguinal lymph node cells were removed and incubated in the presence of either TgP1 (A) or OVA (B) for 4 days *in vitro*. [^{3} H]TdR was added during the last 18h of culture. Background means of quadruplicate wells were: SJL, 5034 cpm; C3H, 822 cpm; B10.BR, 3861 cpm; BALB/c, 1332 cpm, and B10, 595 cpm. SD values did not exceed 15% of the means.

TABLE 4.3: Abrogation of TgP1- specific LNC proliferation by T-cell depletion.

Treatment[a]	TgP1 dose *in vitro* (ug/ml) 20	10
—	3.5 +/- 0.7 (6.1)[b]	2.2 +/- 0.6 (3.9)
anti-Thy1.2 + C'	0.7 +/- 0.4 (1.2)	0.4 +/- 0.1 (0.6)
anti-Thy1.2	5.2 +/- 0.5 (9.2)	4.3 +/- 1.2 (7.6)
C'	8.6 +/- 1.6 (15.2)	9.3 +/- 3.2 (16.4)

[a] TgP1-primed cells from SJL mice were treated with Thy-1.2-specific antibody plus complement as indicated in *Materials and Methods.*

[b] Values represent the cpmx10^3 means of quadruplicate wells. The values in parenthesis correspond to stimulation indices. In the absence of antigen cpm mean of quadruplicate wells was 567.

TABLE 4.4: TgP1 encompasses non-immunodominant T-cell determinants

Antigen *in vivo*[b]	Antigen conc. *in vitro* (nM)[c]	Cell proliferation (stimulation index)[a] after *in vitro* challenge with MTg	RTg	TgP1
MTg	750	.[d]	-	1.3
	75	5.4	2.3	1.2
	7.5	2.6	1.9	1.6
RTg	750	-	-	1.6
	75	2.7	17.8	1.1
	7.5	2.1	14.6	1.1
TgP1	750	1.2	-	11.5
	75	2.0	1.3	2.5
	7.5	1.3	0.9	1.0

[a] In the absence of antigen, cpm means of quadruplicate wells were: MTg-primed LNC = 6,725, RTg-primed LNC=7,257, TgP1-primed LNC= 5,034

[b] SJL mice (4 mice/group) were primed s.c. at the base of the tail with either 75 μg (0.11 nmol) of MTg or RTg, or 218 μg (100 nmol) of TgP1 in CFA. Ten days later their inguinal LNC were stimulated *in vitro* with the indicated antigens and at the doses shown. [^{3}H]TdR was added at 72 hr of culture and the cells were harvested 18 hr later (see *Materials and Methods*).

[c] 750 nM of MTg or RTg corresponds to 495 μg/ml. 750 nM of TgP1 corresponds to 1.6 μg/ml.

[d] (-), not done.

lymphocytes from SJL mice responded significantly to MTg in culture but failed to respond to TgP1 under equimolar (75 nM) or 10-fold higher concentration (750 nM). Similarly, RTg-primed lymphocytes responded strongly to RTg *in vitro*--perhaps due to recognition of foreign (rat) determinants on Tg -- but did not yield detectable responses to TgP1 at 75 or 750 nM. Unresponsiveness was not due to insufficient doses of TgP1 *in vitro* because TgP1-primed lymphocytes responded strongly to TgP1 at the highest dose tested (750 nM). In addition, TgP1 primed lymphocytes did not react to any significant extent with 75 nM MTg, a dose that was clearly stimulatory for MTg-primed cells. Lack of response to MTg was observed at 750 nM (Table 4.4) and at doses between 75 and 750 nM (data not shown), suggesting lack of antigenic stimulation rather than suppressive effects at high antigen dose *in vitro*.

The question of immunodominance was further studied using instead of TgP1-primed LNC, a TgP1-specific T-cell line. The T-cell line was generated from lymph node and spleen cells derived from TgP1-primed mice. It was established after two rounds of *in vitro* stimulation with TgP1 for a total of 24 days (see *Materials and Methods*). The TgP1-specific line responded over a wide range of TgP1 (4.6μM to 0.07μM) *in vitro* but failed to respond to MTg at doses ranging from 1.1μM to 0.018 μM (Fig. 4.5). These results confirmed the findings of bulk cultures and further indicate the non-dominant nature of T-cell determinants delineated by the TgP1 sequence.

4.3.5 TgP1 does not contain serologically immunodominant epitopes but elicits specific antibody responses that do not correlate with peptide-mediated EAT.

In vivo priming with MTg elicited strong MTg-specific IgG responses in all strains tested, but failed to induce IgG cross-reactive with the peptide (Table 4.5), clearly indicating

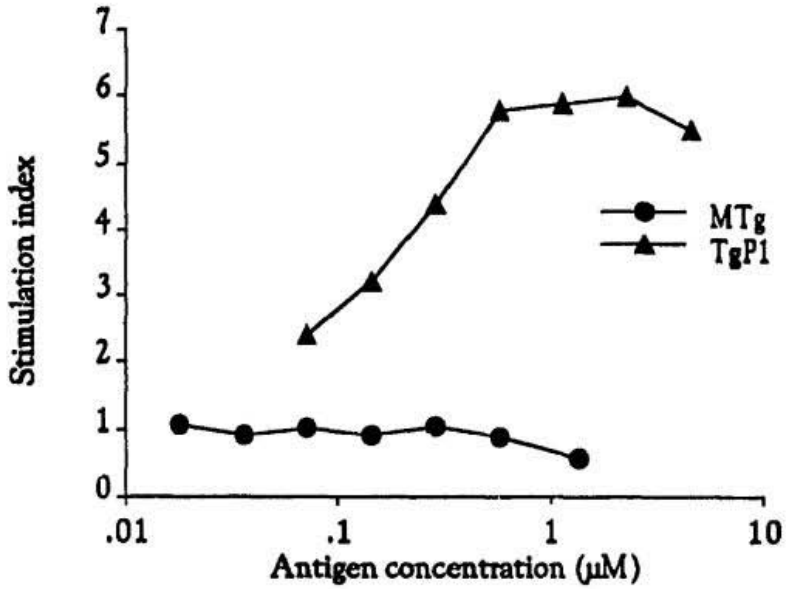


Figure 4.5: TgP1 is a non-dominant T-cell epitope. TgP1-specific T-cell line was generated and tested for specificity to either TgP1 or MTg as described in *Materials and Methods*. Proliferative responses were assessed by [³H] TdR uptake for the last 18 hr of culture. TgP1, MTg starting antigen concentrations were 10 μg/ml and 750 μg/ml respectively. Stimulation index was defined as (cpm in the presence of antigen /cpm in the absence of antigen). Cpm in the absence of antigen was 4540.

TABLE 4.5: TgP1 is not a serologically immunodominant epitope.

Antigen *in vivo*[b]	Strain	O.D. (405 nm) of sera tested *in vitro* against[a] MTg Ser. Dil. 1/256	MTg 1/1,024	TgP1 1/256	TgP1 1/1,024
TgP1	B10	< 0.05	<0.05	<0.05	<0.05
	B10.BR	0.66	0.41	1.39	1.00
	C3H	0.56	0.16	0.88	0.38
	SJL	0.33	0.14	1.50	1.19
	BALB/c	< 0.05	<0.05	0.75	0.52
MTg	B10	1.25	1.21	<0.05	< 0.05
	B10.BR	1.51	1.34	<0.05	< 0.05
	C3H	1.34	0.80	<0.05	< 0.05
	SJL	1.39	1.13	<0.05	< 0.05
	BALB/c	1.15	0.61	<0.05	< 0.05
CFA	B10	<0.08	<0.08	<0.05	<0.05
	B10.BR	<0.08	<0.08	<0.05	<0.05
	C3H	<0.05	<0.05	<0.05	<0.05
	SJL	<0.08	<0.08	<0.05	<0.05
	BALB/c	<0.08	<0.08	<0.05	<0.05

[a] Pooled sera from the mouse groups depicted in Fig. 4.2 were assessed for antigen-specific IgG by ELISA as described in *Materials and Methods*. The data were obtained from full titration curves and are expressed as means of triplicate wells at the indicated dilutions of serum. Standard deviations did not exceed 5% of the mean values.

[b] Mice were primed and boosted with the indicated antigens for EAT induction as described in *Materials and Methods*.

that TgP1 is not a serologically immunodominant epitope. This was not due to a lack of immunogenic B-cell epitopes on TgP1, however, because after TgP1 challenge *in vivo*, strong serologic anti-TgP1 responses were detected in the susceptible (high responder) B10.BR, C3H and SJL strains (Table 4.5). EAT-resistant B10 mice that were unresponsive to TgP1 in proliferative T-cell assays did not yield a peptide-specific antibody response either, suggesting lack of TgP1 immunogenicity in that strain. Interestingly, BALB/c mice that were not significantly susceptible to EAT, and mounted moderate anti-TgP1 proliferative T-cell responses, showed significant IgG responses to TgP1 (Table 4.5). This was not an effect of pooling the BALB/c sera because the six BALB/c mice that did not exhibit mononuclear cell infiltration of the thyroid after priming (Fig. 4.2) were found to be individually reactive to TgP1 in ELISA assays (Fig. 4.6). Such lack of correlation between TgP1-specific titers and EAT suggested that the presence of circulating IgG specific for TgP1 is not pivotal in the induction of thyroid pathology.

4.3.6 Influence of antigen dose and immunization time on the induction of TgP1-specific IgG.

To examine the effect of antigen dose on the production of TgP1-specific IgG, B10.BR mice were immunized twice one week apart with either 5 nmol (11μg) or 50 nmol (109 μg) of TgP1. The ability of those mice to produce TgP1-specific IgG was subsequently tested by ELISA. As shown in Fig. 4.7 the two TgP1 doses tested were not equally immunogenic. The end-titer point of TgP1-specific IgG in the sera of mice primed with the 100 nmol regimen cannot be calculated precisely from the present data, because the titer of that antibody was still significant even at the highest-tested

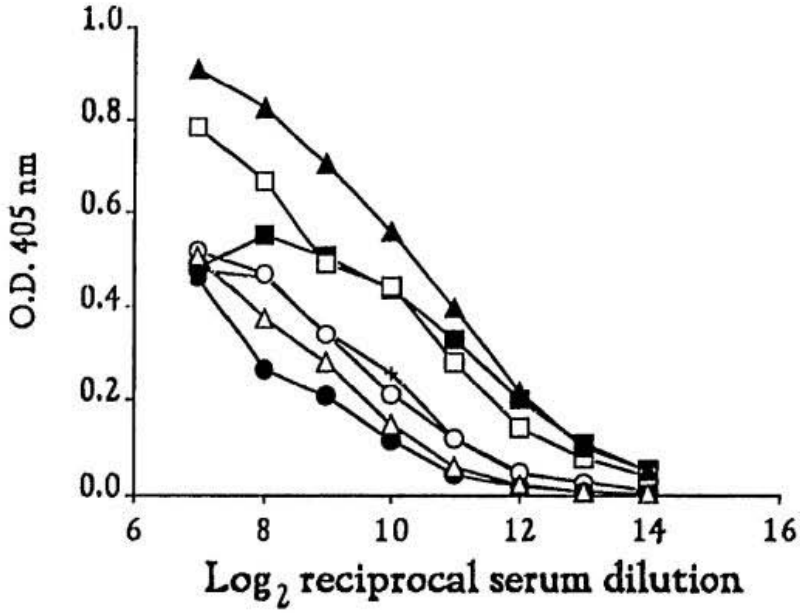


Figure 4.6: Measurment of TgP1-specific IgG in BALB/c mice. Sera from individual mice (see Figure 4.2) were tested for reactivity to TgP1 by ELISA. Each symbol represents a different animal and each point the mean value of triplicate wells at the indicated dilutions of the serum. SD did not exceed 10% of the mean. Pooled sera from the same mice excibited no reactivity to OVA. Values were less than 0.05 at serum dilutions ranging from 1/128 to 1/8192 (data not shown).

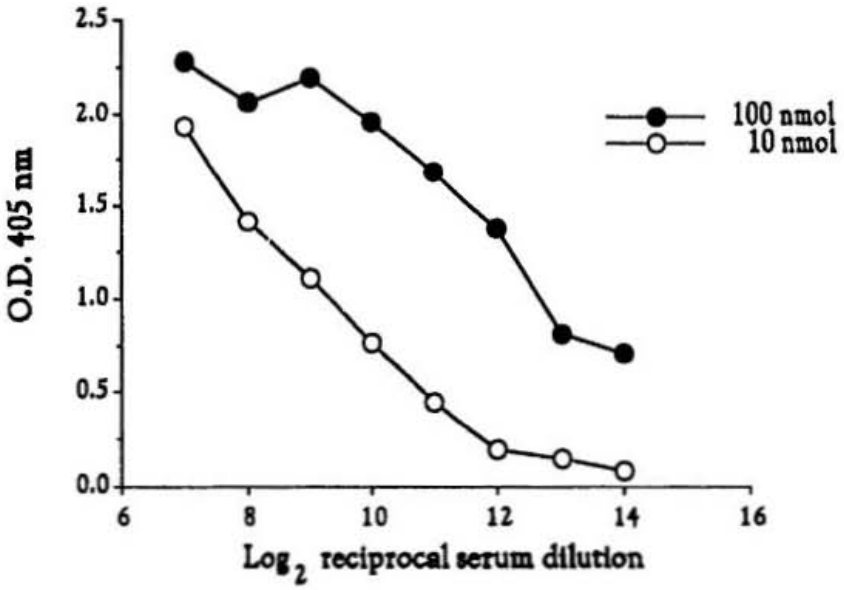


Figure 4.7: Dose response to TgP1 measured as TgP1-specific IgG. Pooled sera from B10.BR mice (3 mice /group) immunized with two doses of either 50 nmol or 5 nmol of TgP1 were tested for TgP1 specific antibodies by ELISA (see *Materials and Methods*). Each point represents the mean of triplicate values. The standard deviation did not exceed the 5% of the mean values.

serum dilution (1/32,768). In approximation, however, it appears that the level of TgP1-specific IgG in the sera of mice receiving the 100 nmol peptide regimen versus those receiving the 10 nmol peptide regimen was at least a 10-fold higher.

To examine the effect of time on the production of TgP1-specific IgG, C3H/HeJ mice were immunized with 100 nmol of peptide and boosted one week later with 50 nmol of TgP1. Pooled sera from such animals were tested for the presence of TgP1 antibodies by ELISA, 21, 27 and 34 days after priming (Fig. 4.8). No clear difference among the TgP1-specific IgG titers was observed at the three time points tested.

4.3.7 TgP1-primed sera cross-react extensively with thyroglobulins from various species.

When TgP1-specific sera were examined for cross-reactivity to MTg, significant responses were observed in sera from EAT-susceptible strains (B10.BR, C3H, and SJL) but not in EAT-resistant BALB/c mice (Table 4.5). TgP1-primed sera from B10.BR, C3H and SJL mice also reacted variably with rat, human, bovine, and porcine Tg (Fig. 4.9). No response was detected against the control antigen OVA (Fig. 4.9). BALB/c sera yielded detectable but, again, consistently low cross-reactive responses to the above Tg. Variability in binding possibly reflected influences of Tg tertiary conformation on determinant accessibility or interference by post-translational modifications such as glycosylation or phosphorylation (J. Charreire, 1989). These data demonstrate the presence of serological determinants delineated by TgP1 on thyroglobulins from various species and support the concept that TgP1 expresses conserved epitope(s).

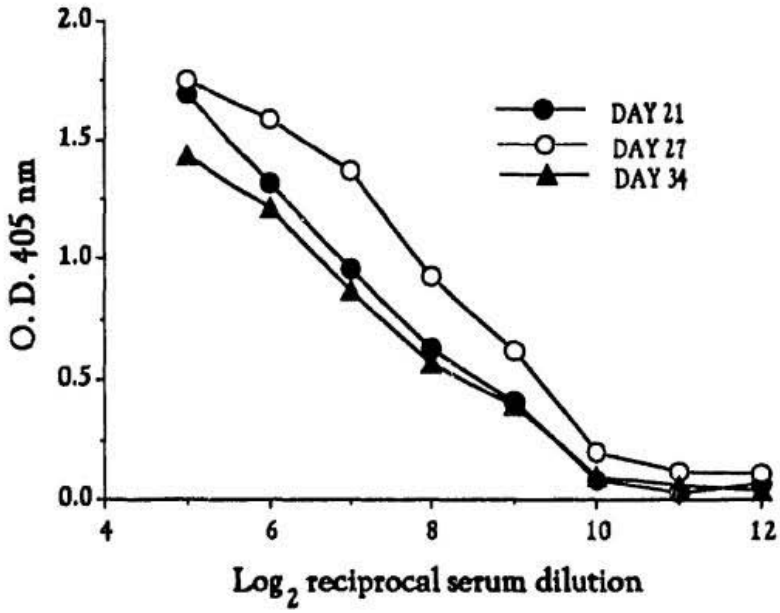


Figure 4.8: TgP1-specific IgG activity in sera harvested 21, 27 and 34 days after immunization. C3H/Hej mice (3 animals/group) were immunized with 100 nmol of TgP1 and boosted one week later with 50 nmol of peptide. Pooled sera from three animals were tested by ELISA for binding to TgP1. Standard deviations did not exceed the 5% of the mean. Pooled sera from the day 21 group did not bind to TgP2 or TgP3 (data not shown). There was no specificity control for the other two groups.

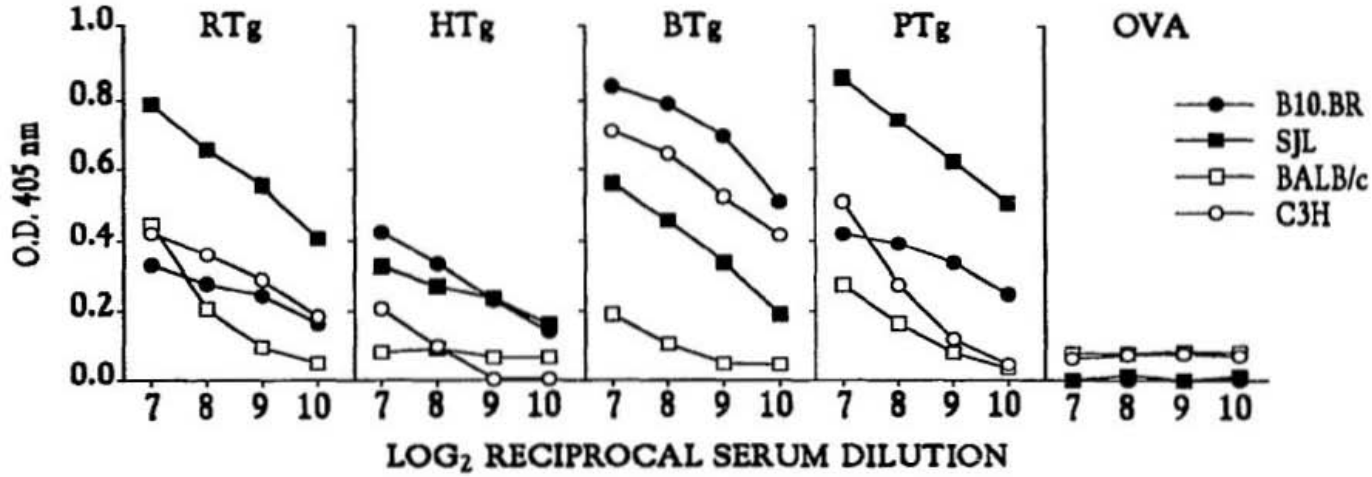


Figure 4.9: Cross-reactivity of TgP1-primed sera to Tgs from various species. Pooled sera from the indicated strains of mice were used. Immunization protocols and the alkaline phoshatase-based ELISA assay are described in *Materials and Methods*.

4.4 DISCUSSION

The present findings demonstrate for the first time the identification of a thyroiditogenic peptide on the Tg molecule through the use of computerized algorithms that predict the presence of T-cell epitopes within a protein sequence (H. Margalit *et al.*, 1987; J. B. Rothbard & W. R. Taylor, 1988). Since EAT induced with intact Tg is under MHC control (see section 1.2.2.1.1) only a few thyroiditogenic peptides must be present within the large Tg molecule. The observation that TgP1-mediated EAT is under similar MHC control suggests that TgP1 is possibly one of the main thyroiditogenic sequences within Tg. The data parallel those from other experimental autoimmune diseases which can be induced with defined autoantigenic T-cell epitopes (M. Kronenberg, 1991). For example, in EAE, induced by direct immunization of the animals with MBP H-2^u has been shown to be a susceptibility haplotype. In two inbred strains carrying the H-2^u haplotype the T-cell responses included pathogenic T cells that were directed to the 1-9 a.a. N-terminal determinant of MBP (S. S. Zamvil et al., 1986). Similarly in collagen-induced arthritis, immunization of rats with type II collagen induces T cells some of which are pathogenic and recognize a single 12 a.a epitope of type II collagen (M. Kronenberg, 1991). In this regard, small Tg fragments, generated by proteolysis, have been isolated with the ability to induce EAT and/or stimulate T-cell responses *in vitro* (D. K. Male *et al.*, 1985; J. Salamero *et al.*, 1987). In one of those studies the primary amino-acid sequence of a 40 amino-acid peptide (localized between residues 1672 and 1711 of HTg has been determined (F40D), that could induce minimal thyroiditis to CBA mice after direct subcutaneous challenge (B. Texier *et al.*, 1992b). In addition, the Tg peptide (2549-2559) STDD(T4)ASFRAL containing T4 at position 2553 was shown to be recognized by Tg-reactive T-cell hybridomas (B. R. Champion *et al.*, 1991). This peptide could not directly induce thyroiditis in CBA/J

mice at doses ranging from 1-25 μg, but LNC primed *in vivo* and boosted *in vitro* with peptide could transfer EAT in naive recipients (P. R. Hutchings *et al.*, 1992). We have also been unable to induce directly mononuclear infiltration of the thyroid in all strains shown in Fig. 4.2 with the 18-mer peptide TDDYASFSRALENATRDY (2550-2567) carrying a tyrosine instead of T4 at position 2553, by using 100 nmol peptide per mouse and 4 mice per strain (see Chapter 8).

The observation that TgP1-specific T-cell proliferation was intense only with primed lymphocytes from EAT-susceptible strains is in agreement with earlier studies that correlated Tg-mediated EAT with T-cell proliferation to MTg in culture (L. L. Simon *et al.*, 1985). The functional profile of the TgP1-specific proliferative T cells remains to be established but it is reasonable to predict that they include Th cells pivotal in the induction of TgP1-specific antibody. Studies with TgP1-specific T-cell clones will be needed to map the minimal T-cell epitope(s) within the TgP1 sequence and to correlate T-cell function and phenotype with epitope recognition (P. Parham, 1991).

The inability of LNC, primed with MTg or RTg *in vivo*, to recognize TgP1 *in vitro* at peptide doses (750 nM) that are clearly stimulatory for TgP1 primed cells suggests that TgP1 consists of non-immunodominant T-cell epitope(s) (Table 4.3). In additional experiments, higher peptide concentrations *in vitro* (up to 3 μM) did not increase specific proliferation of MTg-primed cells. That TgP1 is not immunodominant can also be supported by the observation that TgP1-primed LNC (Table 4.3) or a TgP1 specific T-cell line (Fig. 4.5) from SJL mice can clearly proliferate against TgP1 *in vitro*; they fail to do so against an equimolar dose of MTg (750 nM) which is stimulatory for MTg-primed cells. This latter result has to be interpreted with caution, however, since it is not known whether such a high dose of Tg can be quantitatively processed *in vitro* and to what extent processing of the large Tg molecule can generate sufficient quantity of

relevant peptide *in vitro* to activate specific T cells. Therefore, although TgP1 appears non-immunodominant, its classification as subdominant or cryptic (see section 1.2.4.1) will be easier to establish comparatively, once an immunodominant Tg epitope is identified. The non-immunodominant nature of TgP1 was also suggested by the lack of TgP1-reactivity of MTg-primed sera, and is supported by the fact that TgP1 does not overlap with epitope-bearing sequences on HTg that are known to be recognized by heteroantisera (Q. Dong *et al.*,1989; M. Henry *et al.*, 1990 ; Y. Malthièry *et al.*, 1991).

The lack of TgP1 immunodominance raises the question of how TgP1-primed lymphocytes recognize Tg *in vivo* in order to initiate the autoimmune pathogenic process ($H\text{-}2^s$ is a susceptible haplotype after TgP1 challenge). In that regard, it can be hypothesized that TgP1 is not a product of Tg processing by lymph node antigen-presenting cells but is produced by thyroid proteases. Alternatively, partially digested Tg fragments leaking from the thyroid into the circulation (J. Charreire, 1989; L. J. DeGroot & J. Quintans, 1989) may be processed differently from intact Tg by peripheral antigen-presenting cells. Analogous hypotheses have been proposed about the role of antigen processing on the generation of cryptic but immunopathogenic peptides of the retinal protein IRBP (W. J. Lipham *et al.*, 1991).

Recent studies of Dunn *et al.* on thyroglobulin processing by thyroidal proteases (A. D. Dunn *et al.*, 1991a) have revealed that cathepsins B and L isolated from human thyroids can cleave rabbit Tg at P'_1 residues 2487 and 2490 respectively. This would position TgP1 (N-terminal residue 2495) very close to the N-termini of 27-33 kDa fragments of Tg (A. D. Dunn *et al.*, 1991a) which potentially could enter the circulation and be processed further in the periphery. Since there is high homology at the P_4-P_1 sequences between rat, bovine and human Tg (A. D. Dunn *et al.*, 1991a, R. DiLauro *et al.*, 1985, L. Mercken *et al.*, 1985, Y. Malthièry & S. Lissitzky, 1987), it would be

interesting to test whether limited MTg or RTg digestion with these enzymes would generate immunogenic fragments recognized *in vitro* by TgP1-primed lymphocytes. It is noteworthy that these cysteine endoproteinases along with cathepsin D account for most or all lysosomal endopeptidase acivity found in human thyroid (A. D. Dunn *et al.*, 1991a, A. D. Dunn *et al.*, 1991b) and that the TgP1-containing Tg fragments would include a thyroxine-forming site corresponding to human residue 2553 (A. D. Dunn *et al.*, 1991a).

The induction of TgP1-specific IgG was unexpected since TgP1 was selected by the algorithms only for its potential to bind to MHC antigens. The fact that BALB/c mice mounted TgP1-specific IgG responses but exhibited minimal thyroid infiltration suggests that the presence of antibody alone is not sufficient for EAT induction. This finding is in agreement with earlier studies indicating that Tg-specific antibodies are not necessary for the development of murine EAT (reviewed in J. Charreire 1989; A. O. Vladutiu, 1989; I. Okayasu, 1985 ; L. S. Rayfield *et al.*, 1989). Particularly intriguing, however, was the cross-reactivity of TgP1-induced antisera with MTg. Cross-reactivity could be completely inhibited by TgP1 in competitive inhibition assays and was independent of the tertiary conformation of Tg (Chapter 7) suggesting that cross-reactive IgG was not induced secondarily to TgP1 challenge and was not directed to other determinants on the Tg molecule. These data implicate cross-reactive B cells as possible antigen-presenting cells in TgP1-mediated EAT. Even though B cells are not obligatory in EAT development (section 1.2.3.2), Tg-specific B cells have been shown to process and present self-Tg to T cells (P. Hutchings *et al.*, 1987) and they may precipitate or amplify the disease cascade. Thus, if infectious agents could induce TgP1-reactive B cells through "molecular mimicry" (M. B. A. Oldstone, 1987), priming of MTg-reactive T cells could ensue. Such a mechanism for abrogation of T-cell self tolerance through the

induction of autoreactive B cells has been described by Lin *et al.* with studies on cytochrome c (R-H. Lin *et al.*, 1991). In this regard, it would be worthwhile to examine the role of TgP1-specific B cells in murine EAT and certainly the TgP1-reactivity of both T and B cells in patients with autoimmune thyroid disease.

Recent studies confirmed the thyroiditogenic nature of TgP1 lacking the amino-terminal cystein and carboxyl-terminal tyrosine in an autologous system (B. Balasa & G. Carayanniotis, 1993b). In analogy to the mouse system, TgP1 induced thyroid infiltration in three susceptible rat strains (WKY, F344, WF) and the incidence of thyroiditis correlated well with the T-cell reactivity. The ability of TgP1 to induce thyroid lesions in two different species was attributed to the presence of multiple MHC-binding motifs (Fig. 4.1), a feature that enables TgP1 to bind to various MHC molecules. The last trait in conjunction with the high homology of mouse and rat MHC and the possible sequence identity of TgP1 between those species could explain its pathogenic nature (B. Balasa & G. Carayanniotis, 1993b). Similarly to the mouse system, TgP1 was shown to contain non-immunodominant T-cell epitope(s) because LNC derived from rats after priming with either homologous (RTg) or heterologous (MTg or HTg) Tg failed to respond *in vitro* to TgP1 although they responded significantly to their corresponding antigen. Although no differences in the T-cell reactivity to TgP1 have been observed between the mouse and the rat system, differences in the B-cell reactivity do exist. The TgP1-specific antibodies generally have not been observed in rats and wherever found were low and failed to react with RTg. To explain the apparent differences in the antibody levels it was proposed that mouse and rat immune sera recognize distinct B-cell determinants on TgP1 (B. Balasa & G. Carayanniotis, 1993b). Alternatively, because in the current study TgP1 has been used for immunizations, a part of the antibody could be specific for determinants formed by dimerization of the TgP1

amino-terminal cysteine. In view of the short length of TgP1 and the lack of use of carrier-peptide conjugates for peptide coating during ELISA assays, dimerization of TgP1 might also increase its coating efficiency. Evidence supporting both of the hypotheses stated above is provided in chapter 7. In addition, to explain the lack of reactivity of rat immune sera derived from TgP1-primed animals with rat Tg *in vitro*, it has been speculated that Tg might adopt a tertiary conformation that renders the TgP1 epitope non-accessible to TgP1-specific IgG (B. Balasa & G. Carayanniotis, 1993b).

Identification of additional EAT-inducing peptides will aid in our understanding of the peptide-MHC interactions regulating thyroiditis and will afford detailed studies on the heterogeneity of T-cell receptors recognizing such peptide-MHC complexes. The feasibility of immunotherapies based on MHC-blocking peptide analogs or anticlonotypic antibodies will then be more easily assessed.

CHAPTER 5

H-2E^k EXPRESSION INFLUENCES THYROIDITIS INDUCTION BY THE THYROGLOBULIN PEPTIDE (2495-2511)[1]

5.1 SUMMARY

This study attempts to map the H-2 region(s) responsible for EAT induction using a defined Tg peptide TgP1(2495-2511) and intra-H-2 recombinant mice. We find that, within the susceptible H-2^k haplotype, H-2E expression is a necessary requirement for EAT induction but it requires concomitant expression of H-2A and/or H-2K products. Lack of H-2E expression is associated with loss of proliferative LNC responses to TgP1 *in vitro* but does not affect the TgP1-specific IgG response. The loss of proliferative LNC responses might not be due to the lack of proliferation of H-2E-restricted T cells but rather of H-2A-restricted cells whose proliferation depends upon the help of the former cell population. These results contrast with earlier findings with Tg-mediated EAT that have assigned a major regulatory role to the H-2A^k region and may reflect processes detectable only after challenge with thyroiditogenic peptides but not with intact Tg.

5.2 INTRODUCTION

EAT induced with Tg and adjuvant has been studied extensively in mice as a model of HT (J. Charreire, 1989). Early studies, involving 33 inbred strains of mice representing

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[1] Part of this chapter has been published as: Chronopoulou E. & Carayanniotis G. 1993. H-2E^k expression influences thytoiditis induction by the thyroglobulin peptide (2495-2511). *Immunogenetics*. Vol. 38: 150-153.

11 different H-2 haplotypes, correlated EAT with MHC genes (A. O. Vladutiu & N. R. Rose, 1971a). Strains of the H-2^k, H-2^s, or H-2^q haplotypes appeared to be particularly susceptible to the disease (good responders), whereas H-2^b or H-2^d strains were relatively resistant (poor responders). The first experiments which addressed EAT induction in intra-H-2-recombinant mice mapped susceptibility within the centromeric side of the H-2 complex (V. Tomazic *et al.*, 1974). Further studies on Tg-mediated EAT localized control of susceptibility in the H-2A region of the H-2^k haplotype (K. W. Beisel *et al.*, 1982a). Genetic influences on EAT development have also been attributed to the K or the D region of the H-2 complex (R. Maron & I. R. Cohen, 1979; A. Ben-Nun *et al.*, 1980; R. Maron & I. R. Cohen, 1980; Y. M. Kong *et al.*, 1979) and to non-H-2 genes (K. W. Beisel *et al.*, 1982b).

An important role for the induction of EAT has also been assigned to T cells (J. Charreire, 1989). The primary response of mouse lymphocytes to syngeneic thyroid epithelial cells was found to be under similar H-2 control as EAT susceptibility (J. Salamero & J. Charreire, 1983a) and proliferation was attributed to recognition of I-A region products (J. Salamero & J. Charreire, 1983b). The combined evidence that Tg-mediated EAT is under H-2 control and that Tg-specific T cells are responsible for the disease suggests that the large Tg molecule (MW=660 kDa) harbors only a limited number of thyroiditogenic peptides that can be recognized by MHC-restricted T cells.

We have recently identified such a thyroiditogenic epitope (TgP1) localized at a.a. 2495-2511 of rat Tg (see Chapter 4). TgP1 is strikingly similar to Tg as far as three main EAT criteria are concerned: it induces lymphocytic infiltration of the thyroid, it is recognized by T cells, and it elicits IgG antibodies which cross-react with Tgs from various species. Furthermore, it causes EAT with a genetic pattern analogous to that of Tg-mediated EAT: B10.BR or C3H (H-2^k) and SJL (H-2^s) mice are susceptible,

BALB/c ($H\text{-}2^d$) mice are relatively resistant and B10 ($H\text{-}2^b$) mice are completely resistant and immunologically unresponsive to TgP1. These similarities prompted us to examine whether EAT mediated by the 17-mer TgP1 would also be controlled by products of the H-2A region in a manner analogous to EAT caused by intact Tg.

5.3 RESULTS

5.3.1 I-E region products are critical for thyroiditis induction

Intra-H-2 recombinant mice with B10 background were challenged s.c. with 100 nmol TgP1 in CFA and boosted s.c. 3 weeks later with 50 nmol peptide in IFA. EAT induction was monitored five weeks after the first challenge, as previously described (Chapter 4). All B10.BR but no B10 mice developed EAT (Table 5.1) confirming our earlier observation that this model of EAT is under direct H-2 control. All seven B10.A and five out of six B10.A(2R) mice were also susceptible, suggesting that the H-2D region does not control TgP1-mediated EAT within the $H\text{-}2^k$ haplotype. Interestingly, B10.A(4R) mice that express $H\text{-}2K^k$ and $H\text{-}2A^k$ but not $H\text{-}2E^k$ molecules were completely resistant to the disease. This indicated that $H\text{-}2E^k$ expression is necessary for the induction of lymphocytic infiltration. The presence of $H\text{-}2E^k$ molecules alone is not sufficient for EAT susceptibility, however, since B10.A(5R) mice that express $H\text{-}2K^b$, $H\text{-}2A^b$ and $H\text{-}2E^k$ molecules are also resistant. These data indicate a possible synergism or interaction between $H\text{-}2E^k$-restricted and $H\text{-}2K^k$- or $H\text{-}2A^k$-restricted TgP1-specific T cells in the development of the disease. Evidence that $H\text{-}2A^k$ and $H\text{-}2E^k$-restricted T cells are being generated against TgP1 *in vivo* is presented in the section 5.3.4. In addition, V. P. Rao and colleagues have isolated TgP1-specific clonal populations in the

TABLE 5.1: EAT induction by Tg peptide (2495-2511) in intra-H-2 recombinant strains [a]

	H-2 loci					Infiltration index						.Mice
Strain	K	Aβ	Aα	Eβ	Eα	D	0	0.5	1	2	3	4	with EAT
B10	*b*	*b*		*(b)*¥	-†	*b*	7	0	0	0	0	0	0/7
B10.BR	*k*	*k*		*k*	*k*	*k*	0	2	2	1	2	0	7/7
B10.A	*k*	*k*		*k*	*k*	*d*	0	2	2	1	2	0	7/7
B10.A(2R)	*k*	*k*		*k*	*k*	*b*	1	0	0	3	1	1	5/6
B10.A(4R)	*k*	*k*		*(k)*¥	-†	*b*	7	0	0	0	0	0	0/7
B10.A(5R)	*b*	*b*		*b*	*k*	*d*	6	0	0	0	0	0	0/6

[a] Reagents, method of immunization and scoring of mononuclear cell infiltration of the thyroid were as described in *Meterials and Methods*. For all strains, female mice were purchased from the Jackson Laboratory, Bar Harbor, ME and were used at 7 weeks of age.

¥ not expressed on the cell surface

† No gene product.

form of T-cell hybridomas, which are either H-2A or H-2E restricted (V. P. Rao *et al.*, 1994). On the other hand, a possible requirement for H-2K^k expression is compatible with the notion that H-2K product expression on the thyroid regulates EAT induction within the H-2^k haplotype (R. Maron & I. R. Cohen, 1979; A. Ben-Nun *et al.*, 1980; R. Maron & I. R. Cohen, 1980).

5.3.2 Correlation between proliferation and susceptibility to thyroiditis.

The clearcut profile of EAT observed in the intra-H-2-recombinant mice (see table 5.1) allowed us to test whether peptide-specific proliferative T-cell responses correlate with susceptibility. Mice from each strain were challenged s.c. at the base of the tail with 50 nmol TgP1 and, ten days later, inguinal LNC were tested for proliferation to TgP1 *in vitro* (see *Materials and Methods*). Data from two experiments (Table 5.2) indicate that only LNC from the susceptible strains B10.BR, B10.A and B10.A(2R) responded consistently and significantly (S.I. values >2.8) to the peptide *in vitro*. Among the resistant strains, B10.A(4R) and B10 mice that are H-2E$^-$ were unresponsive, whereas B10.A(5R) that are H-2E$^+$ showed significant proliferation in one of the two experiments. These observations support our earlier data indicating that strains of various H-2 haplotypes, susceptible to TgP1-mediated EAT, exhibit strong proliferative T-cell responses to TgP1 *in vitro* (Chapter 4) and are analogous to findings with Tg-mediated EAT (I. Okayasu *et al.*, 1981).

TABLE 5.2: TgP1-primed LNC from susceptible strains proliferate strongly to TgP1 *in vitro*

		Proliferative response to antigens *in vitro* (cpm +/- S.D.)/1000 (S.I.)[b]					
	H-2 region	Exp.1			Exp.2		
Strain[a]	K A E D	None	TgP1	PPD	None	TgP1	PPD
B10	*b b b b*	14.0 +/- 1.7	18.5 +/- 1.3 (1.3)	34.5 +/- 1.3 (2.5)	6.5 +/- 0.4	5.6 +/- 1.5 (0.9)	17.5 +/- 2.8 (2.7)
B10.BR	*k k k k*	22.1 +/- 2.7	92.1 +/- 0.7 (4.2)	45.5 +/- 3.0 (2.0)	5.3 +/- 0.2	14.9 +/- 3.9 (2.8)	33.0 +/- 4.5 (6.3)
B10.A	*k k k d*	4.5 +/- 0.9	18.9 +/- 1.7 (4.2)	20.0 +/- 1.8 (4.5)	ND[c]	ND	ND
B10.A(2R)	*k k k b*	7.4 +/- 0.4	43.8 +/- 6.3 (5.9)	28.2 +/- 1.2 (3.8)	11.0 +/- 1.8	35.7 +/- 4.7 (3.2)	35.7 +/- 5.0 (3.2)
B10.A(4R)	*k k b b*	6.4 +/- 1.6	10.3 +/- 2.2 (1.6)	54.6 +/- 3.2 (6.6)	2.8 +/- 0.7	2.3 +/- 0.6 (0.8)	18.1 +/- 1.5 (5.4)
B10.A(5R)	*b b k d*	17.3 +/- 0.5	49.1 +/- 1.5 (2.8)	34.8 +/- 1.3 (2.0)	5.3 +/- 1.0	7.4 +/- 1.8 (1.4)	22.3 +/- 3.5 (4.2)

[a] Mice were primed s.c. at the base of the tail with 50 nmol TgP1 in CFA. Ten days later, the draining inguinal LNC were cultured in triplicate wells with TgP1 (20 µg/ml) or PPD (10 µg/ml) for 4 days. Tritiated thymidine (1 µCi/well) was added during the last 18 hr of culture. Cells were harvested on filters and incorporated radioactivity was measured in a scintillation counter. Data are obtained from full antigen titration curves. No response was observed against OVA (not shown).

[b] The S.I. is defined as described in *Materials and Methods.*

[c] not done

5.3.3 Correlation between TgP1-induced Tg-reactive antibody and EAT susceptibility.

When the sera from mice shown in Table 5.1 were analyzed for the presence of TgP1-specific IgG by ELISA, all strains responded strongly except for B10 mice that were unresponsive (Fig. 5.1A). No correlation, therefore, exists between the presence of TgP1-specific IgG and EAT susceptibility as previously reported with other strains (Chapter 4). Interestingly, peptide-specific IgG cross-reacted with MTg only in the susceptible B10.BR, B10.A and B10.A(2R) mice (Fig. 5.1B). Thyroglobulin binding could be completely inhibited by free TgP1 (see Chapter 7). This finding suggests that different strains recognize distinct serological epitopes on TgP1, some of which are accessible by antibodies on native Tg, but it is not clear at present to what extent cross-reactive antibodies contribute to EAT induction.

5.3.4 T cells recognize TgP1 in association with I-A- and I-E-region products.

To determine the MHC-restriction profile of the proliferative LNC in the H-2^k haplotype, C3H/HeJ (H-2^k) mice were immunized with 50 nmol TgP1 and ten days later their inguinal LNC were cultured *in vitro* with TgP1 in the presence of anti-I-A^k and anti-I-E^k or both MoAbs (Fig. 5.2). In control wells a MoAb specific for influenza A nucleoprotein (anti-NP) was added. As shown in Fig 5.2, addition of I-A^k-specific MoAb inhibited the proliferation of LNC in a dose-dependent manner suggesting the presence of TgP1-specific I-A^k-restricted cells. In contrast to the above result, addition of I-E^k-specific MoAb inhibited the LNC proliferation completely at all doses tested. The last finding was surprising, but it is likely due to the antibody-doses used for

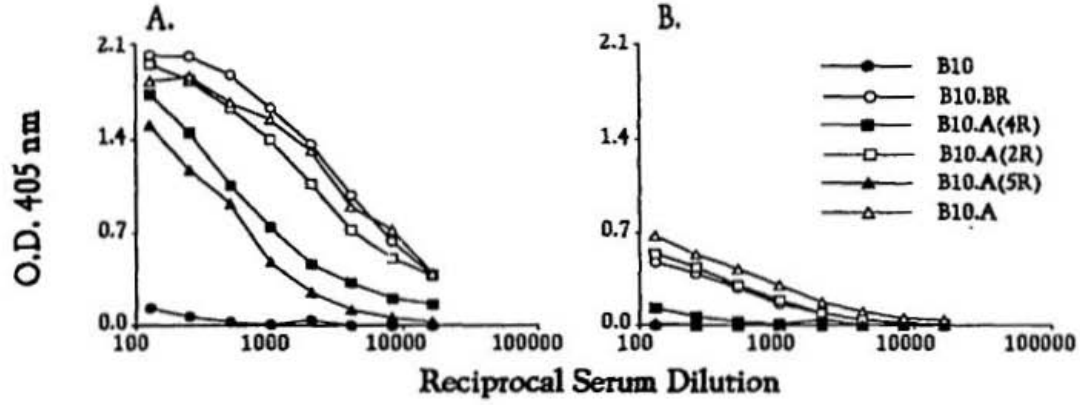

Figure 5.1: Measurement of the serological response to TgP1 in intra H-2 recombinant mice. **A.** IgG response to TgP1; **B.** IgG responses to mouse Tg. Pooled sera (day 35) from the mice of Table 5.1 were used in an alkaline phosphatase-based ELISA as described in *Materials and Methods*.

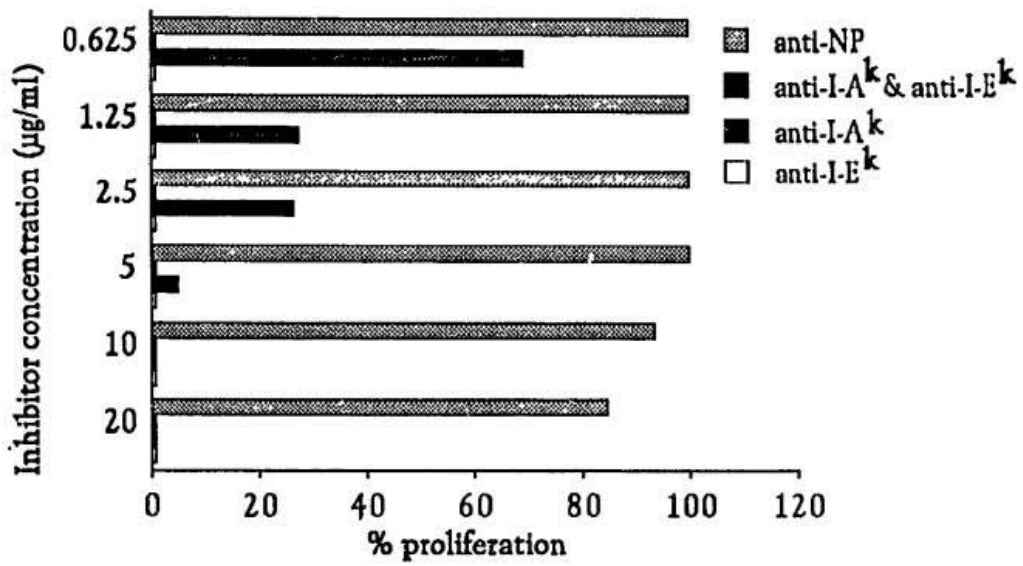

Figure 5.2: MHC-restriction of the *in vitro* LNC-proliferative response in H-2^k(C3H/HeJ) mice. TgP1-primed LNC from 3 mice were cultured *in vitro* for 4 days (see *Materials and Methods*) with the I-A^k-specific (TIB 92), I-E^k-specific (HB 32) MoAbs or with a control antibody to influenza nucleoprotein (HB 65). MoAb concentration varied between 0.625 µg/ml to 20 µg/ml. TgP1 concentration *in vitro* used was 10 µg/ml. In the absence of antigen the mean value of quadruplicate wells was 23,420 cpm. Anti-I-E^k constantly blocks proliferation.

blocking of LNC proliferation. Evidence to support the last speculation comes from a recent study in which the same anti-I-E^k specific MoAb has been used to block TgP1-specific recognition by an I-E^k-restricted T-cell hybridoma clone (V. P. Rao *et al.*, 1994). In that study, the authors have shown that only anti-I-E^k MoAb doses less than 1µg/ml allow TgP1 recognition by the hybridoma clone. In this study, however, even an antibody dose of 0.625 µg/ml could completely inhibit the proliferation of I-E^k-restricted cells. The above observation is striking because one would expect that a certain level of proliferation would exist due to proliferation of I-A^k-restricted cells. To explain the lack of proliferation of LNC inhibited by I-E^k-specific MoAb we could hypothesize that I-A^k cells, in order to proliferate in culture, require helper signals from I-E^k cells. In favor of that hypothesis are recent data showing that LNC restricted to the I-A^k-binding epitope of TgP1 do not proliferate (V. P. Rao *et al.*, 1994). To address synergisms between different cell subsets *in vitro*, however, it is necessary to generate clonal populations of TgP1-specific, H-2A- and H-2E- restricted cells. The blocking of TgP1-induced LNC proliferation *in vitro* by I-A^k- or I-E^k-specific MoAbs was specific for the antibodies used because LNC proliferation was not affected by a MoAb specific for influenza A nucleoprotein. The present data suggest that TgP1 delineates sequences recognized by T cells is the context of H-2A^k and H-2E^k molecules.

5.4 DISCUSSION

Our finding that H-2E^k region products are critical in the development of TgP1-mediated EAT is in apparent discrepancy with previous studies that show a strong H-2A^k influence in EAT induction with intact Tg (K. W. Beisel *et al.*, 1982a). Also no effect of the H-2D region on peptide-mediated EAT was discerned in contrast to earlier

data documenting influence of this region on Tg-mediated EAT (Y. M. Kong *et al.*, 1979). Two possible explanations may account for our findings. First, if EAT-regulating T-cell clones recognize the *majority* of Tg epitopes in the context of H-2A^k, the influence of H-2E^k molecules in the presentation of individual pathogenic peptides such as TgP1 may be masked when Tg is used for *in vivo* challenge. Similarly, it is conceivable that Tg epitopes other than TgP1 may be presented in the context of H-2D. This concept needs to be tested with other Tg epitopes such as the ones that were recently described causing EAT in H-2^k mice (P. R. Hutchings *et al.*, 1992; B. Texier *et al.*, 1992b). Second, TgP1-mediated EAT may differ significantly from Tg-mediated EAT in antigen processing and presentation requirements. TgP1 contains non-immunodominant determinants (Chapter 4), and it may be prevented from binding to E molecules by higher-affinity Tg epitopes. Moreover, binding of Tg to A molecules via dominant determinants may reduce the possibility of an adjoining non-dominant E restricted determinant such as TgP1 to form a productive class II ligands (H. Deng *et al.*, 1993). Alternatively, TgP1 may not be generated *in vivo* after processing of exogenous Tg by professional APC. Instead, it might be generated by thyroid proteases and expressed in the context of class I or class II products on the thyroid gland where it would be recognized by specific T cells activated in the periphery by TgP1 and adjuvant. In nature, TgP1-reactivity could be elicited via molecular mimicry of this peptide by proteins derived from various pathogens, such as the reovirus, which is known to cause mouse thyroiditis (reviewed in Y. Tomer & T. F. Davies, 1993; J. Srinivasappa *et al.*, 1988).

The association of TgP1-specific reactivity with H-2E expression was surprising since E molecules are known to control suppression of the immune response to protein

antigens (C. N. Baxevanis *et al.*, 1982; D. Oliveira *et al.*, 1985) although responses to synthetic polypeptides such as GLT are channelled through the E molecule (N. Ishii *et al.*, 1981). In nature several A^+E^- strains have been observed but not A^-E^+. Thus, it was speculated that E and A molecules are not functionally equivalent. Recently, A^-E^+ mice were produced by crossing the Eα16 line which carries an Eα transgene on a non-MHC chromosome with the $A^0\beta$ line, that carries a mutated Aβ gene (D. Cosgrove *et al.*, 1992). A and E molecules were equally capable of restoring the $CD4^+$ cells in the periphery, of promoting maturation of $CD4^+CD8^-$ cells in the thymus, of permitting efficient CTL generation and of reestablishing normal antibody production to T-cell dependent antigens compared to class II-deficient animals. The last result is in agreement with this study and provides further evidence of EAT regulation by E molecules, a feature previously assigned to A molecules. Such regulation may involve TgP1-specific T cells recruited from subsets such as Tcrb-V10b$^+$ or Tcrb-V6$^+$ T cells that are known to be positively selected in H-2E$^+$ mice (K. Tomonari *et al.*, 1992; H. R. MacDonald *et al.*, 1988). On the other hand, our data demonstrate that TgP1-mediated EAT requires H-2E expression only within the susceptible $H\text{-}2^k$ haplotype since H-2E$^-$ SJL mice do develop EAT and specific T-cell responses after challenge with TgP1 and adjuvant (Chapter 4). It is, therefore, difficult to reconcile the present findings with results from other experimental models such as the NOD mice which spontaneously develop diabetes (S. Makino *et al.*, 1980) and thyroiditis (N. F. Bernard *et al.*, 1991). Although such comparisons cannot be made, it is noteworthy to mention that our data are in agreement with recent studies in IDDM. Early studies on the development of insulitis and diabetes in NOD mice suggested that H-2E expression prevents rather than enhances the autoimmune insulitis (H. Nishimoto *et al.*, 1987). However, a recent report in which I-E$^+$ F1 mice carrying a single dose of NOD-MHC in the NOD

background were used, shows that I-E expression does not prevent development of insulitis or diabetes. In fact I-E^+ F1 mice developed insulitis and cyclophosphamide-induced diabetes at 5-12 months (mo) of age. IDDM could also develop spontaneously in those mice aged 9-17 mo (P. L. Podolin *et al.*, 1993).

In this study it is shown that TgP1 is recognized in the context of both A^k and E^k molecules, suggesting that it encompasses at least two distinct or overlapping T-cell epitopes. This assumption has been confirmed in both the rat (B. Balasa & G. Carayanniotis, 1993b) and the mouse system of TgP1-induced thyroiditis (V. P. Rao *et al.*, 1994). In the rat system, the TgP1-specific proliferation of LNC derived from strains susceptible to EAT (F344, WF, WKY) was significantly blocked by MoAbs directed to either RT1-B (I-A equivalent) or RT1-D (I-E equivalent) antigens. (B. Balasa & G. Carayanniotis, 1993b). In addition, clonal populations in the form of T-cell hybridomas have been obtained that recognize TgP1 in the context of I-A^k and I-E^k molecules (V. P. Rao *et al.*, 1994). Two of those TgP1-specific hybridoma clones (4A2 and 4A12) have been successfully used as tools to identify among truncated TgP1 peptides the minimal thyroiditogenic epitopes within TgP1. As an outcome of that study TgP1 was shown to delineate two nanomeric overlapping E^k- and A^k-binding epitopes with a.a. coordinates (2496-2504) and (2499-2507) respectively (V. P. Rao *et al.*, 1994). Mice directly challenged by the I-E-binding epitope developed lesions equivalent to those obtained after challenge with TgP1. The last finding suggests the I-E^k-binding epitope as the main thyroiditogenic sequence within TgP1 (V. P. Rao *et al.*, 1994) and explains why TgP1-induced EAT is under the control of E^k genes (current study).

Our findings suggest that the I-E-restricted cell proliferation is not consistent (Table 5.2). Several speculations can be made about the role of I-E-restricted cells. They might assist in the development of a serological response to TgP1 or they may be helping in the

activation of effector cells (I-A restricted ?). Further studies using clonal H-2A and H-2E restricted T-cell populations are required to clarify their individual roles in disease pathogenicity.

CHAPTER 6

DISTINCT GENETIC PATTERN OF SUSCEPTIBILITY TO THYROIDITIS IN MICE INDUCED BY A NOVEL NON-DOMINANT THYROGLOBULIN PEPTIDE (2695-2713)[1]

6.1 SUMMARY

EAT, induced by Tg and adjuvant, is MHC-controlled and dependent on Tg-reactive T cells, but the immunopathogenic T-cell epitopes on Tg remain mostly undefined. We report here the thyroiditogenicity of a novel rat Tg peptide (TgP2; corresponding to human Tg amino acids 2695-2713), identified by algorithms as a site of putative T-cell epitope(s). TgP2 causes EAT in SJL (H-2^s) but not in C3H or B10.BR (H-2^k), BALB/c (H-2^d) and B10 (H-2^b) mice. This reveals a new genetic pattern of EAT susceptibility since H-2^k mice are known to be high responders (susceptible) after Tg challenge (see paragraph 1.2.2.1.1). Following *in vivo* priming with TgP2, only T cells from SJL mice proliferated significantly and consistently to TgP2 *in vitro*, whereas TgP2-specific IgG was observed in all strains tested. Adoptive transfer of TgP2-primed SJL LNC to naive syngeneic recipients induced a pronounced mononuclear infiltration of the thyroid, which was more extensive than that observed after direct peptide challenge. TgP2 is non-immunodominant, since priming of SJL mice with RTg did not consistently elicit T-cell responses to TgP2 *in vitro*. Similarly, a TgP2-specific line failed to proliferate to MTg *in vitro* at doses between 100 µg/ml to 10µg/ml. The data support the notion that Tg epitopes need not be either iodinated or immunodominant in

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[1] Part of the results presented in this chapter has been published as: Carayanniotis G., Chronopoulou E. & Rao V. P. 1994. Distinct genetic pattern of mouse susceptibility to thyroiditis induced with a novel thyroglobulin peptide. *Immunogenetics*, Vol. 39:21-28.

order to cause severe thyroiditis and that the genetic pattern of the disease they induce can be distinct from that of Tg-mediated EAT.

6.2 INTRODUCTION

The delineation of defined pathogenic T-cell epitopes within the large Tg molecule (homodimeric MW=660 kDa) remains a high-priority objective in EAT, an animal prototype of HT. Such knowledge can facilitate experimental design at the clonal level and will greatly aid in our understanding of the immunoregulation of EAT and the human disease. Recent studies in this area have yielded fruitful results by means of two approaches: First, cloned T-cell hybridomas generated after challenge with intact Tg, and reactive with immunodominant determinants have been used to screen enzymatically derived Tg fragments of decreasing size or synthetic Tg peptides for T-cell epitope mapping. With this method, the nanomeric Tg peptide 2551-2559, containing T4 at position 2553, was characterized as a minimal T-cell epitope (B. R. Champion *et al.*, 1991), and it was subsequently shown that T cells reacting to this peptide could adoptively transfer EAT to naive recipients (P. R. Hutchings *et al.*, 1992). Also, through the use of cytolytic T-cell hybridomas, a 40 a.a. sequence was identified from the central, non-hormonogenic Tg region, which induced thyroid infiltration in mice (B. Texier *et al.*, 1992b).

Second, we have shown that the use of algorithms predicting T-cell epitopes within a protein sequence (H. Margalit *et al.*, 1987; J. B. Rothbard & W. R. Taylor, 1988) can be a successful alternative method for the identification of pathogenic Tg sequences. With this approach we have identified a non-dominant 17-mer Tg peptide (a.a. 2495-2511; TgP1) that induces EAT in mice (Chapters 4, 5) and rats (B. Balasa & G.

Carayanniotis, 1993b), is recognized by T cells, and elicits IgG responses that cross-react with Tgs from various species. Furthermore, TgP1 causes EAT in mice with a genetic pattern similar to that of Tg-mediated EAT: H-2^k and H-2^s mice are susceptible, H-2^d mice are relatively resistant, and H-2^b mice are completely resistant to EAT and unresponsive after TgP1 challenge. In the present report, we have similarly used several mouse strains to examine the immunogenicity and pathogenicity of a novel Tg peptide (a.a. 2695-2713; TgP2), which was delineated by predictive algorithms as a putative site of T-cell epitope(s).

6.3 RESULTS

6.3.1 TgP2 has features of MHC-binding peptides

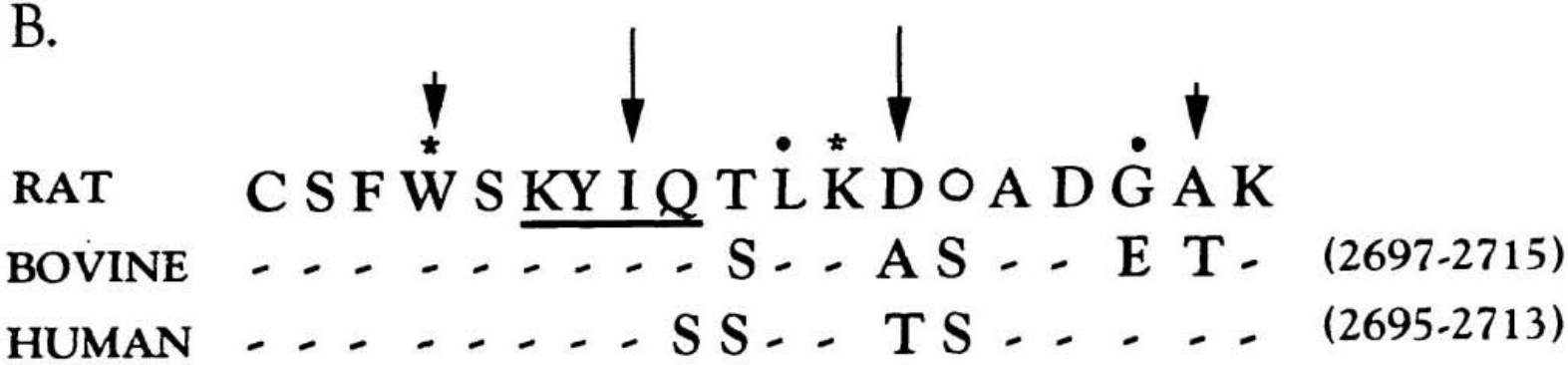
Scanning data of the known portion of the RTg sequence (R. Di Lauro *et al.*, 1985) by the AMPHI (H. Margalit *et al.*, 1987) and Rothbard and Taylor "tetramer motif" (J. B. Rothbard & W. R. Taylor, 1988) algorithms strongly suggested the 18-mer peptide TgP2 with the amino-terminal Cys2695 as a potential site for T-cell epitope(s). This peptide is located very close to the carboxy-terminus of Tg (Fig. 6.1A), is mostly conserved among the rat, cattle, and human Tg sequences (Fig. 6.1B), and, due to the deletion of Ser2708 within RTg, it was assigned the coordinates of the human 19-mer sequence 2695-2713, since the complete RTg sequence is unknown. TgP2 carries an amphipathic segment (a.a. 2698-2712, amphipathic score 39.6, l=11) and a tetramer motif starting at position 2700 (Fig. 6.1B). It also contains two sequences with an MHC-binding motif (C. M. Hill *et al.*, 1991) in which the hydrophobic a.a. Ile2702 and Leu2705 are four residues apart from the small a.a. Asp2707 and Gly 2711, respectively.

Figure 6.1: A. The carboxy terminal 348 amino acids of human Tg (Y. Malthiery & S. Lissitzky, 1987; single letter code; numbering does not include the 19-residue leader sequence). It shows the relative positions (*bold underlined*) of : thyroiditogenic human 9mer sequence (2551-2559) containing thyroxine instead of tyrosine at position 2553 (P. Hutchings et al., 1992) and homologous positions of thyroiditogenic rat peptides TgP1 (a.a. 2495-2511) (Chapter 4) and TgP2 (2695-2713; present report). **B. Primary amino acid sequence of TgP2 and homologies with its cattle and human counterparts.** *Underlined*, tetramer motif (j. B. Rothbard & W. R. Taylor, 1988); *short arrows*, amphipathic segment according to the AMPHI program (amphipathic score 39.6, l=11) (H. Margalit et al., 1987); *long arrows* or •, pairs of a.a. obeying an MHC-binding motif (C. M. Hill et al., 1991); *, a.a. in agreement with an E^k-binding motif (J. Leighton et al., 1991); (-) identical a.a. between rat and cattle or human sequences; O, a.a. deletion.

A.

2401	AAVISHERAQQQAIALAKEVSCPMSSSQEVVSCLRQKPANVLNDAQTKLL	2450
2451	AVSGPFHYWGPVIDGHFLREPPARALKRSLWVEVDLLIGSSQDD**GLINRA**	2500
2501	**KAVKQFEESRG**RTSSKTAFYQALQNSLGGEDSDARVEAAATWYYSLEHST	2550
2551	**DD(T4)ASFSRA**LENATRDYFIICPIIDMASAWAKRARGNVFMYHAPENYGHG	2600
2601	SLELLADVQFALGLPFYPAYEGQFSLEEKSLSLKIMQYFSHFIRSGNPNY	2650
2651	PYEFSRKVPTFATPWPDFVPRAGGENYKEFSELLPNRQGLKKAD**CSFWSK**	2700
2701	**YISSLKTSADGAK**GGQSAESEEEELTAGSGLREDLLSLQEPGSKTYSK	2748

B.



The positioning of the two aromatic a.a. Phe2697 and Trp2698, seven residues away from Lys2706 also reveals an H-2E^k-binding motif (J. Leighton *et al.*, 1991). TgP2 also carries two motifs that are observed in 50-80% of good DR-binding peptides. Those motifs are composed of the aromatic residue Phe1697 and hydrophobic residue Ile1702 in position 1 followed respectively by the non-charged and relatively small residues Ile1702 and Ala1709 in position 6 and the relatively hydrophobic residues Lys1705 and Ala1712 in position 9 (D. O'Sullivan *et al.*, 1991) (see Fig 6.2). TgP2 also encompasses binding motifs that are found in self peptides that are released from class II HLA-DR1 (Ile1702-Ala2712) (H. Kropshofer *et al.*, 1992), DR 3/DRw52 (Tyr2701-Thr2704) (R. M. Chicz *et al.*, 1993; A. Geluk *et al.*, 1994), DR8 (Ile1702-Lys2706) (R. M. Chicz *et al.*, 1993) and DR2a/DR2b molecules (Ile1702-Lys2713) (R. M. Chicz *et al.*, 1993) (Fig. 6.2). Analysis of the TgP2 sequence via the PHYSCHEM program of PCGENE revealed a list of physicochemical characteristics that are reviewed in Table 6.1. The estimation of pI, and half-life *in vivo* for TgP2 were based on algorithms described in section 4.3.1.

6.3.2 TgP2 induces thyroiditis in H-2^s but not H-2^k mice

EAT induction was monitored in B10, B10.BR, SJL, C3H and BALB/c mice (eight mice per group) primed and boosted with TgP2 as described in Table 6.2. Small but quite distinct foci of mononuclear infiltration of the thyroid (Fig. 6.3 A, C) were observed in 6 of 8 SJL (H-2^s) mice, a strain commonly susceptible to Tg-mediated EAT (A. O. Vladutiu & N. R. Rose, 1971a). Mice from the other strains did not exhibit detectable infiltration of the thyroid (Table 6.2), including H-2^k mice such as C3H and B10.BR which are also known to be susceptible to Tg-mediated EAT. The split in TgP2-mediated EAT between the s and k haplotypes was unexpected and demonstrates

Figure 6.2: TgP2 carries several DR-binding motifs. The numbers correspond to the human TgP2 homologue. *Short arrows* and ∞, DR-binding motifs according to D. O'Sullivan et al., (1991). *Long arrows*, delineate a DR3/DRw52-binding motif (R. M. Chicz et al., 1993; A. Geluk et al., 1994). * , a.a in agreement with a DR2a/2b-binding motif (R. M. Chicz et al., 1993).O , a.a in agreement with a DR1-binding motif (H. Kropshofer et al., 1992). •, a.a obeying an DR8-binding motif (R. M. Chicz et al., 1993)

TABLE 6.1: Physicochemical characteristics of TgP2 §

Molecular weight (MW)	2061
Isoelectric point (pI)	9.04
Half-life *in vitro*	
mammalian reticulocytes	1.2 hr
Half-life *in vivo*	
yeast	>20 hr
Escherichia coli	>10 hr

§ Physichochemical characteristics of TgP2 according to the PHYSCHEM program of PCGENE (see section 4.3.1).

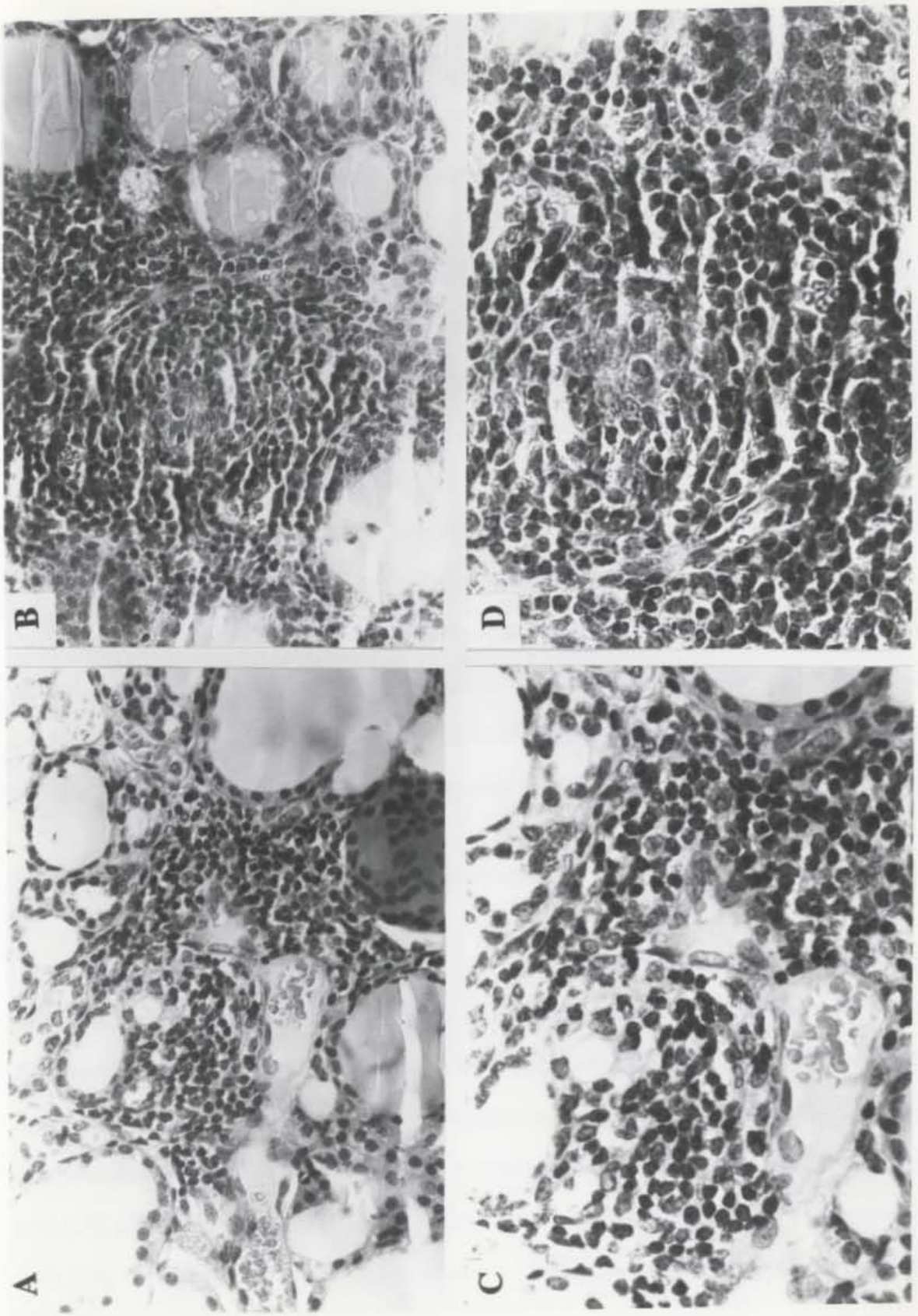
TABLE 6.2: EAT induction by TgP2 in various mouse strains [a]

Strain	H-2 haplotype	Infiltration index[b] 0	0.5	1	2	3	4	Mice with EAT
B10	b	8	0	0	0	0	0	0/8
B10.BR	k	8	0	0	0	0	0	0/8
SJL	s	2	0	6	0	0	0	6/8
BALB/c	d	8	0	0	0	0	0	0/8
C3H	k	8	0	0	0	0	0	0/8

[a] Mice were challenged s.c. with 100 nmol TgP2 and three weeks later they were boosted s.c. with 50 nmol of peptide.

[b] Lymphocytic infiltration of the thyroid was assessed five weeks after the initial challenge as described in *Materials and Methods.*

Figure 6.3 A-D: TgP2-induced mononuclear cell infiltration of the thyroid in SJL mice. Thyroid sections were derived from animals either challenged directly with TgP2 [A(X250), C(X400)], or i.p. adoptively transferred with $2X10^7$ syngeneic TgP2-specific LNC[B(X250), D(X400)].


B
D
A
C

that the classification of a strain as EAT-susceptible or resistant can differ significantly, depending on whether intact Tg or individual Tg-peptides are used.

6.3.3 TgP2 elicits consistently proliferative T-cell responses only in SJL mice

Mice from the above strains were subsequently challenged s.c. with 100 nmol TgP2 and ten days later, the proliferative response of primed inguinal LNC was determined *in vitro* against TgP2. TgP1 and OVA were used *in vitro* as antigen controls. Results from three independent experiments (Table 6.3) show that only LNC from SJL mice responded consistently and significantly to TgP2 *in vitro*, exhibiting S.I. values >2.2. The response was specific since background proliferation was observed against TgP1 or OVA (not shown). LNC from H-2^k (C3H and B10.BR) or H-2^b (B10) mice were not activated by TgP2 *in vitro*, whereas LNC from H-2^d (BALB/c) mice responded in one of three experiments (S.I. = 2.8). Treatment of SJL LNC with anti-Thy 1.2 plus complement but not with complement or antibody alone, abrogated completely the proliferative response (Table 6.4) suggesting that the responding cells were T cells.

6.3.4 TgP2 is not a dominant T-cell determinant.

Since sensitization to dominant T-cell epitopes occurs consistently after challenge with the intact protein antigen *in vivo* (G. Gammon *et al.*, 1987), we sought to determine whether priming of SJL mice with RTg would lead to a detectable TgP2-specific LNC reactivity *in vitro*. In three experiments, shown in Table 6.5, RTg-primed lymphocytes responded strongly to RTg *in vitro* but failed to respond to equimolar (0.1 and 0.01 μM) concentrations of TgP2 that were clearly stimulatory for TgP2-primed cells (Exp. 4). In one of the three experiments (Exp. 2) RTg-primed LNC responded to

TABLE 6.3: Proliferative response of LNC to TgP2 *in vitro* (mean cpm +/- S.D.) /1000 (S.I.) [a]

	H-2	Exp. 1		Exp. 2		Exp. 3	
Strain	region	Medium	TgP2	Medium	TgP2	Medium	TgP2
B10	b	3.3 +/- 0.4	5.4 +/- 1.0 (1.7)	1.7 +/- 0.5	3.2 +/- 1.2 (1.8)	0.5 +/- 0.1	1.0 +/- 0.1 (1.9)
B10.BR	k	1.9 +/- 0.7	2.5 +/- 0.3 (1.3)	1.4 +/- 0.5	1.6 +/- 0.5 (1.2)	0.2 +/- 0.1	0.4 +/- 0.1 (1.8)
SJL	s	21.4 +/- 3.3	98.6 +/- 5.8 (4.4)	7.0 +/- 1.5	17.4 +/- 4.2 (2.5)	7.6 +/- 1.2	16.8 +/- 3.4 (2.2)
C3H	k	13.5 +/- 2.1	15.2 +/- 2.5(1.1)	15.1 +/- 2.9	21.1 +/- 7.5 (1.4)	0.2 +/- 0.1	0.1 +/- 0 (0.8)
BALB/c	d	7.8 +/- 1.6	21.8 +/- 5.6 (2.8)	3.6 +/- 0.9	3.6 +/- 1.0 (1.0)	1.4 +/- 0.1	1.7 +/- 0.2 (1.2)

[a] Mice were primed s.c. at the base of the tail with 100 nmol TgP2 and ten days later inguinal LNC were cultured in triplicate wells with TgP2 for 4 days (see *Materials and Methods*). Data are obtained from antigen titration curves and denote the response to 1.2 μM TgP2 *in vitro*. Culture with OVA (20 μg/ml) or another thyroiditogenic peptide of Tg (TgP1, 1.2 μM) yielded a background response (data not shown). The S.I., is defined as described in *Materials and Methods*.

TABLE 6.4: Abrogation of TgP2-specific LNC proliferation by T-cell depletion.

Treatment[a]	TgP2 dose *in vitro* (µg/ml) 20	10
—	16.0 +/- 2.3[b] (8.2)	14.7 +/- 3.1 (7.5)
anti-Thy1.2 + C'	0.3 +/- 0.09 (0.1)	0.4 +/- 0.1 (0.2)
anti-Thy1.2	43.3 +/- 3.6 (22.2)	32.0 +/- 6.2 (16.4)
C'	31.1 +/- 5.0 (16.0)	28.3 +/- 0.3 (14.5)

[a] TgP2 primed LNC from SJL mice were treated with anti-Thy-1.2 antibody plus complement as indicated in *Materials and Methods.* Controls included cells treated with either anti-Thy 1.2 antibody or complement alone.

[b] Values represent the mean cpmx10^3 of quadruplicate wells. The values in parenthesis correspond to stimulation indices. In the absence of antigen the cpm mean of quadruplicate wells was 1950.

TABLE 6.5: TgP2 does not encompass dominant T-cell determinants [a]

Proliferative responses of inguinal LNC expressed as stimulation index [b]

Exp. no.	Antigen used for challenge		Antigen concentration (μM) *in vitro*			
	in vivo	*in vitro*	10	1	0.1	0.01
1	RTg	RTg	ND[c]	ND	5.7	3.5
		TgP2	1.1	1.0	1.3	1.1
2	RTg	RTg	ND	ND	22.5	17.6
		TgP2	6.5	4.4	1.5	1.4
3	RTg	RTg	ND	ND	24.4	22.3
		TgP2	0.9	0.7	0.8	1.3
4	TgP2	TgP2	11.2	10.7	5.8	2.8

[a] SJL mice were challenged s.c. at the base of the tail with 75 μg RTg (Exp. 1-3) or 103 μg TgP2 (Exp. 4) in 0.1 ml complete Freund's adjuvant/phosphate buffered saline emulsion. Ten days later, their inguinal LNC were stimulated for 4 days in vitro as shown. In the absence of added antigen *in vitro*, background cpm were: Exp. 1, 2,423; Exp. 2, 9,325; Exp. 3, 5,023; Exp. 4, 7,713. 0.1 μM RTg =66 μg/ml; 0.1 μM TgP2=0.21 μg/ml.

[b] Stimulation index: cpm in the presence of antigen/cpm in the absence of antigen.

[c] not done.

10-100-fold higher concentrations of TgP2 *in vitro*. This suggested that TgP2 was not adequately processed in RTg.

The dominance versus crypticity question for TgP2 was re-examined using a TgP2 specific T-cell line generated from TgP2-specific cells which were derived from both the lymph nodes and the spleens of mice 8 days after priming with TgP2. Briefly, the TgP2-specific cells were stimulated *in vitro* twice with antigen and feeders and the TgP2-specific T-line was selected after 24 days of *in vitro* culture. The TgP2-specific T-cell line responded strongly to TgP2 *in vitro* but it failed to respond to MTg at doses ranging between 0.151μM (100μg/ml) to 0.018 μM (12.5 μg/ml) (Fig 6.4). The inability of the TgP2-specific T-cell line to respond to such doses of MTg was interpreted as follows: First, the counterpart sequence of TgP2 on MTg might differ to such an extent that it fails to activate the T-cell line. Although TgP2 is not highly conserved among those species whose Tg a.a. sequence is available (Fig. 6.1B), this explanation is rather implausible. Second, TgP2 might not be an immunodominant sequence of MTg. This hypothesis is favored both by results obtained from bulk cultures (Exp. 1, 3, Table 6.5) and by data obtained from a TgP2-specific T-cell hybridoma (6E10) (G. Carayanniotis *et al.*, 1994). Although the 6E10 T-cell hybridoma could strongly respond to TgP2 *in vitro* it failed to respond to MTg- or RTg-pulsed APC suggesting that TgP2 is not generated *in vitro* after MTg or RTg processing. The former data strongly indicate the non-dominant nature of TgP2.

6.3.5 TgP2-primed LNC adoptively transfer EAT to naive recipients

Donor mice were s.c. challenged at the base of the tail with 50 nmol TgP2 in CFA or CFA alone, and ten days later their inguinal LNC were obtained and cultured in the presence of TgP2 or con A. Three days later, TgP2-activated LNC were transferred i.p.

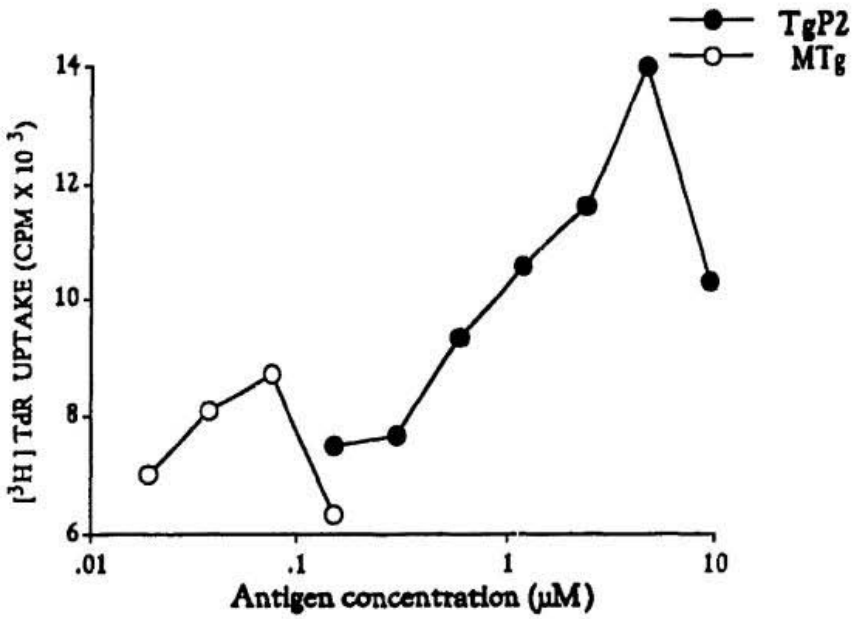


Figure 6.4: Dose response curve of a TgP2-specific T-cell line. The TgP2-specific line was generated as described in *Materials and Methods* and its ability to proliferate *in vitro* for 4 days to either MTg or TgP2 was tested in a thymidine uptake assay. TgP2 and MTg starting concentrations were 20μg/ml (9.7 μM), and 100 μg/ml (0.15 μM) respectively. In the absence of antigen the mean value of quadruplicate wells was, 6854.

to six normal syngeneic recipients (2×10^7 cells/animal), whereas five control animals received an equivalent number of LNC primed *in vivo* with CFA and stimulated *in vitro* with con A. Fourteen days after transfer, severe thyroiditis affecting almost 50% of one or both lobes of the thyroid gland (Fig. 6.3 B, D) was observed in mice that received TgP2-specific LNC (Table 6.6). No lymphocytic infiltration was observed in the control animals. To ascertain that the cells transferring EAT were indeed TgP2-specific, a portion of the cells that were transferred was left in culture for an additional day and their ability to proliferate to TgP2 was assessed by [^{3}H]-TdR uptake. To exclude the possibility that the CFA-primed cells were dead at the time of transfer their ability to proliferate to con A was also tested. TgP2-primed cells could strongly proliferate to TgP2 (Table 6.7). Similarly, CFA-primed cells were strongly responsive *in vitro* to con A suggesting that their inability to transfer thyroiditis was not due to reduced viability. TgP2-specific IgG was not detected in the sera of recipients with EAT (Fig. 6.5) suggesting that antibody does not play a significant role in the development of the EAT lesions.

6.3.6 TgP2-specific IgG cross-reacts with heat-denatured but not intact thyroglobulins.

Sera from all strains of Table 6.2, obtained at the time of thyroid gland removal (day 35), expressed high titers of TgP2-specific IgG antibody (Fig. 6.6). Thus, in several strains, despite the absence of EAT and the lack of detectable TgP2-specific proliferative T cells *in vitro*, TgP2 appeared clearly immunogenic at the level of the B-cell response. In a subsequent assay, the time dependence for the development of such a response was studied. Mice immunized with TgP2 as indicated in the legend of Figure 6.7, were sacrificed at different time points (21, 27, and 34 days after priming) and the level of

TABLE 6.6: Adoptive transfer of EAT by TgP2-specific LNC in SJL mice.

Priming of adoptively transferred cells		Infiltration index [a]						Mice with
In vivo	*In vitro*	0	0.5	1	2	3	4	EAT
TgP2 + CFA	TgP2	0	0	1	0	5	0	6/6
CFA	con A	5	0	0	0	0	0	0/5

[a] Inguinal LNC were primed as shown and transferred i.p. into syngeneic normal recipients. Thyroid glands were collected 14 days later and the thyroid pathology was assesed as previously described (see *Materials and Methods*).

TABLE 6.7: Specificity test of lymphocytes used for adoptive transfer experiments.

Proliferative responses of inguinal lymph node cells (mean cpm+/- SD)/1000. (S.I.)

Antigen [a] *in vivo*	Ag dose *in vitro* (µg/ml)	Antigen *in vitro* (µg/ml) TgP2	con A
TgP2	10	109.5 +/- 2.6 (*7.9*)	*ND* [b]
	5	105.8 +/- 2.3 (*7.6*)	-
	2.5	110.9 +/- 4.9 (*8.0*)	-
	1.25	100.2 +/- 6.6 (*7.2*)	-
	-	13.9 +/- 4.6	-
CFA	4	*ND*	74.4 +/- 8.2 (*8.9*)
	2	-	77.8 +/- 6.4 (*9.3*)
	1	-	60.3 +/- 4.4 (*7.2*)
	0.5	-	42.3 +/- 8.7 (*5.1*)
	-	-	8.3 +/- 1.5

[a] SJL mice were immunized s.c. at the base of the tail with 50 nmol of TgP2 in CFA or CFA/PBS. Ten days after priming, the inguinal LNC were cultured in the presence of either TgP2 or con A. Proliferation was assessed by [^{3}H]TdR uptake (see *Materials and Methods*).

[b] not done.

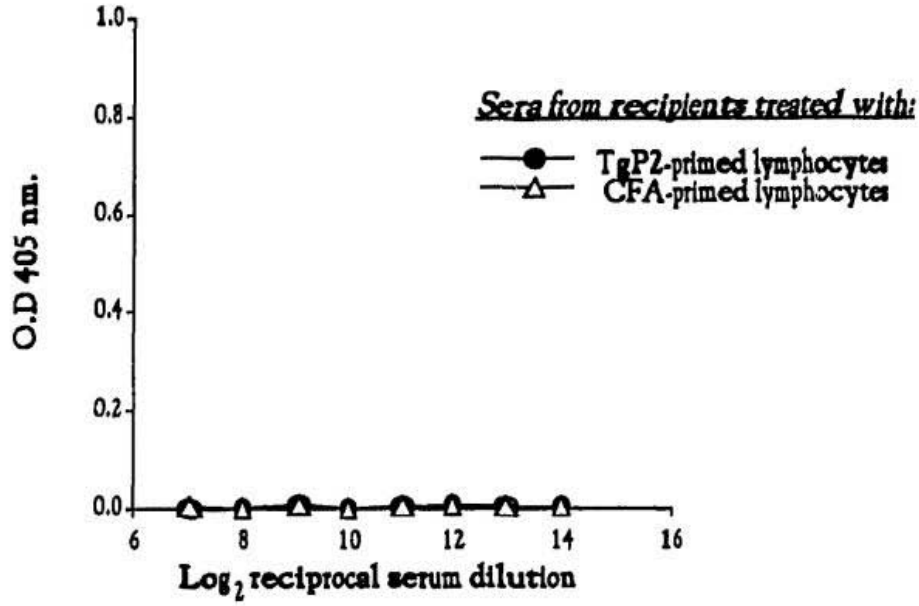


Figure 6.5: Lack of TgP2-specific IgG responses in recipient mice adoptively transferred with TgP2-primed lymphocytes. Pooled sera from SJL mice (see Table 6.4) (day 14) were tested for TgP2-specific antibodies by ELISA. Standard deviation did not exceed 4.5% of the mean.

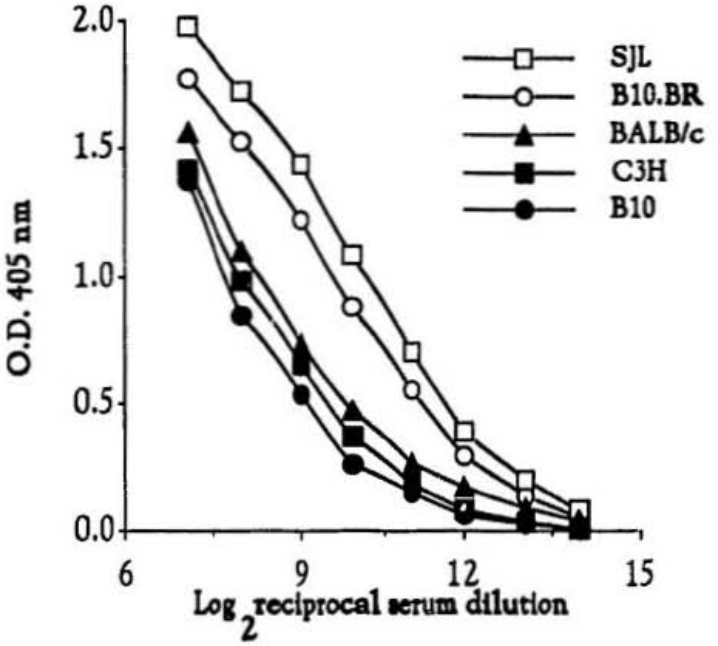


Figure 6.6: Measurement of TgP2-specific IgG in the sera of various mouse strains. Pooled sera (day 35) from four mice of the indicated strains, assessed by ELISA based on alkaline phosphatase. OD values are expressed as means of triplicate wells at the indicated dilutions of serum. SD did not exceed 4% of the mean values.

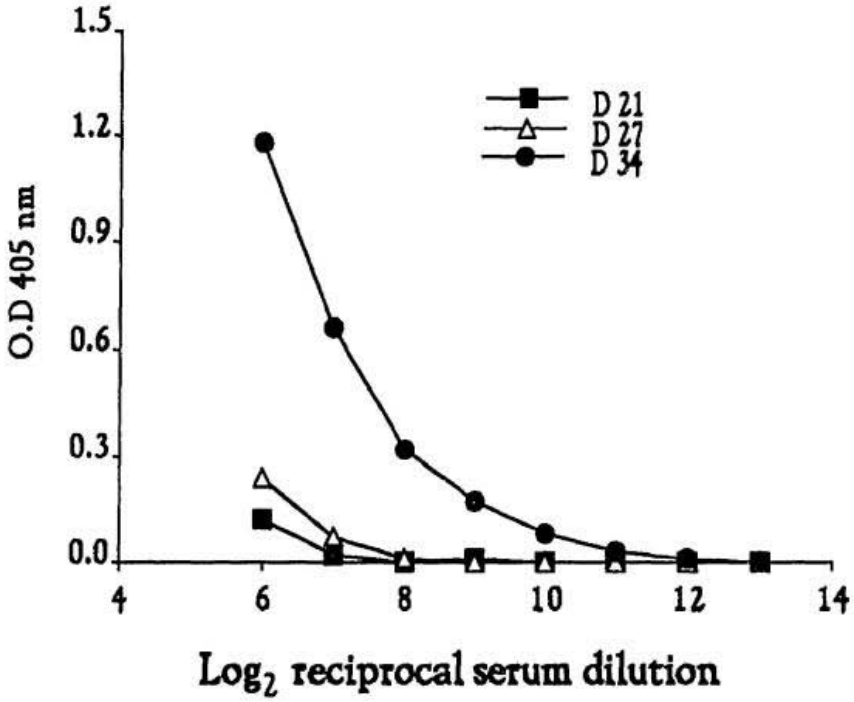


Figure 6.7: TgP2-specific IgG activity in sera harvested 21, 27 and 34 days after immunization. C3H/Hej mice were immunized with 100 nmol TgP2 and boosted one week later with 50 nmol of peptide. Pooled sera from three animals was tested by ELISA (see *Materials and Methods*) for binding to TgP2. Each point represents the mean value of an assay in triplicate. Standard deviations did not exceed 5% of the mean.

TgP2-specific antibodies was determined by ELISA (Fig. 6.7). Interestingly, no detectable antibodies were demonstrated in the sera of those mice at 21 or 27 days after priming, although such antibodies were present in the serum on day 34. Based on the previous findings, no direct correlation between the appearance of TgP2-specific antibodies and EAT establishment could be made, because the C3H/HeJ strain is resistant to TgP2-induced EAT. When we examined the reactivity of TgP2-primed sera from SJL mice against intact thyroglobulins from various species, cross-reactive IgG responses were not observed (Fig. 6.8A). However, heat-denaturation of thyroglobulins enhanced specific binding significantly (Fig. 6.8B), an outcome compatible with the IgG response being directed against a linear Tg determinant.

6.4 DISCUSSION

In the field of EAT, genetic susceptibility as well as immunoregulatory mechanisms have been commonly studied using intact Tg as an immunogen (J. Charreire, 1989). The valid assumption that EAT results from an autoreactive response to immunodominant Tg determinants has led to intense efforts to map T-cell epitopes on Tg, using cloned T-cell hybridomas that have been induced after challenge with the intact protein (see section 1.2.4.1). At the same time, the use of predictive algorithms has allowed us to identify two Tg peptides-(2495-2511, TgP1), (Chapters 4, 5) and (2695-2713, TgP2), (the current chapter)-that elicit T-cell responses and cause severe thyroiditis. Moreover both peptides encompass T-cell epitopes which are defined as non-dominant. This definition is based on the observation that these epitopes are not consistently recognized by Tg-primed T cells and are not detected by peptide-specific T cells during Tg-processing *in vitro* (see section 1.2.4.1). For example, Tg-processing by

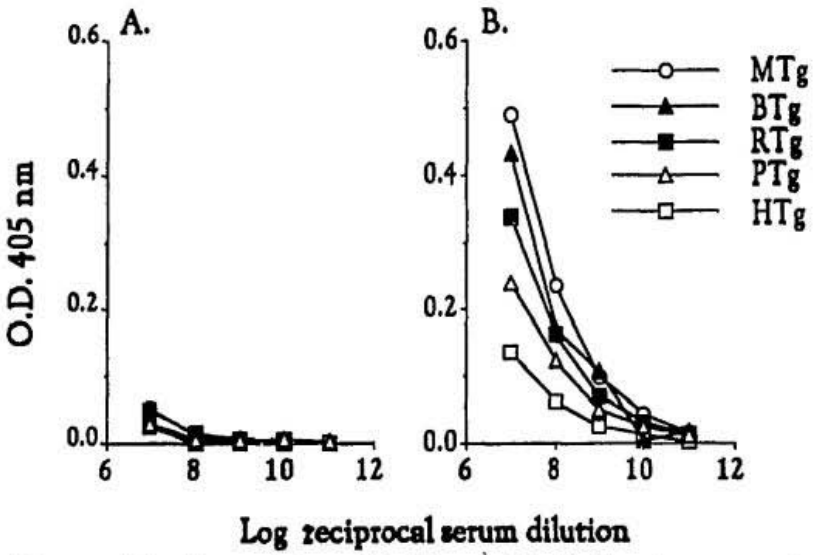


Figure 6.8: Reactivity of TgP2-specific IgG to intact and heat-denatured Tg from various species. Pooled TgP2-primed sera from four SJL mice (Table 6.2) were tested to intact **(A)** or heat-denatured **(B)** mouse, rat, human, cattle or pig Tg. An alkaline-phosphatase-conjugated goat anti-mouse IgG was used in the ELISA. OD values represent means of triplicate wells. SD did not exceed 5% of the mean values.

splenic cells reportedly generates the dominant peptide 2551-2559 recognized by the CH9 hybridoma *in vitro* (B. R. Champion *et al.*, 1991), but it does not create the TgP2 peptide that stimulates the TgP2-specific cloned T-cell hybridoma, 6E10 (G. Carayanniotis *et al.*, 1994). The lack of generation of TgP2 by splenic cells is also evident from their inability to trigger proliferation of a TgP2-specific line at doses as high as 100 μg/ml (Fig. 6.4).

Initial studies with TgP1 and TgP2 have yielded results that do not always conform with observations made in Tg-mediated EAT. Thus, we have previously shown that, in H-2^k mice, EAT mediated by TgP1 requires expression of H-$2E^k$ determinants (Chapter 5), whereas such a requirement has not been reported for Tg-mediated EAT. Also in the present study, mice carrying the "classic" susceptibility H-2^k haplotype do not develop EAT after TgP2 challenge. It is possible that in the absence of intramolecular competition with higher affinity peptides, individual non-dominant Tg epitopes exhibit distinct MHC-binding and pathogenic properties. A haplotype classified as a low responder (resistant) to Tg may appear susceptible when challenged with a Tg peptide and alternatively, as shown here a high responder (susceptible) haplotype may not respond to an individual pathogenic epitope. In fact, the inability of TgP2 to induce EAT in H-2^k mice could be due to the expression of I-E molecules that might exert a protective role. I-E molecules could capture the determinant that is pathogenic in the context of I-A molecules and thus abolish its pathogenic effect (H. Deng *et al.*, 1993). SJL mice, however, develop the disease because they lack expression of I-E molecules due to a deletion of the promoter of the Eα gene. Such a mechanism of abrogation of autoimmunity has been postulated to operate in transgenic Ea^d NOD mice that are protected from IDDM (H. Nishimoto *et al.*, 1987; T. Lund *et al.*, 1990).

To explain the lack of immunodominance of TgP2 we could speculate that its thyroiditogenic effect is masked by other epitopes that bind with high affinity to the MHC. An alternative explanation could be that TgP2 is not generated at all after Tg-processing, although some data (Table 6.5, Exp. 2) do not favour this hypothesis. Studies with rabbit Tg (A. D. Dunn *et al.*, 1991a) have suggested that thyroidal cathepsins D and L cleave Tg at positions 2643 and 2657, respectively, generating 15-22 kDa carboxy-terminal fragments that contain TgP2. Such fragments may leak from the thyroid and could form better substrates for TgP2 generation by APC *in vivo* than Tg provided exogenously to APC *in vitro*. The fact that EAT is observed after adoptive transfer of TgP2-primed LNC strongly suggests that TgP2 is generated either in intrathyroidal dendritic cells or in thyrocytes following degradation of Tg by thyroid proteases. The latter hypothesis will be further substantiated if TEC can be found to present TgP2 directly to T cells.

At present we do not know whether the distinct genetic pattern of EAT, observed with non-dominant Tg epitopes, represents a new model of thyroiditis and, if so, whether such a model mimics events that precipitate the human disease. Non-dominant Tg peptides may contribute to the pathogenesis within the context of two main theories of thyroiditis induction; namely, Tg iodination (R. S. Sundick *et al.*, 1987) and infection (J. Srinivasappa *et al.*, 1988). Compelling evidence (B. R. Champion *et al.*, 1987a; B. R. Champion *et al.*, 1992; R. S. Sundick *et al.*, 1987; B. R. Champion *et al.*, 1991) suggests that iodination enhances Tg immunogenicity, but it is not yet clear whether this enhancement results solely from the creation of dominant (and/or) iodinated T-cell epitopes or involves novel (non-dominant?) epitopes as well. Such epitopes could be generated if iodinated Tg offers new substrate sites for antigen processing (L. Lamas & S. H. Ingbar, 1978; F. Fouchier *et al.*, 1983). TgP2 contains a tyrosyl residue at position

2701 but this is not one of the iodotyrosine sites identified in HTg (L. Lamas *et al.*, 1989). It is, therefore, unlikely that it constitutes a "donor" tyrosine during hormonogenesis (R. DiLauro *et al.*, 1985). As shown in chapters 4, 5, the current chapter and other studies (B. Texier *et al.*, 1992b), Tg determinants do not need to be iodinated in order to cause EAT. Those studies are in agreement with the previously observed induction of EAT in CBA mice with cells activated *in vitro* with T4-poor Tg (reviewed in D. C. Rayner *et al.*, 1993). In the context of infection, non-dominant Tg peptides may be recognized via molecular mimicry with proteins from various pathogens such as the reovirus that causes mouse thyroiditis (J. Srinivasappa *et al.*, 1988) and induces class II expression on thyroid cells (D. S. Neufeld *et al.*, 1989). They may also cross react with proteins from microbial flora that predispose for thyroiditis (W. J. Penhale & P. R. Young, 1988). Regardless of the etiology, the involvement of non-dominant Tg epitopes in the development of thyroid pathology requires careful consideration in view of recent evidence that demonstrates "spreading" of autoreactivity from dominant to cryptic epitopes of autoantigens (P. V. Lehmann *et al.*, 1992) and tolerance induction to immunodominant but not to subdominant and cryptic epitopes of an autoantigen (R. Cibotti *et al.*, 1992).

The TgP2 sequence lies within a larger HTg determinant (a.a. 2644-2730) that was previously reported (Q. Dong *et al.*, 1989) to be recognized by rabbit antibodies but not by sera from patients with thyroid disease. In contrast, screening of HTg fusion fragments by rabbit antisera did not detect TgP2-containing determinants in another study (M. Henry *et al.*, 1990). While it remains to be established whether the human analog of TgP2 is recognized by antibodies or T cells from patients with thyroid disease, priming of mice with homologous intact Tg does not elicit significant TgP2-specific IgG (data not shown), whereas following challenge with TgP2, mouse sera cross-react

detectably to denatured but not intact MTg, as shown in this study. These data suggest that accessibility to the TgP2 epitope by antibodies may vary, depending on the nature of the immunogen and the system of study used.

The presence of TgP2-specific IgG in mouse strains which do not exhibit T-cell proliferative responses or EAT after TgP2 challenge suggests distinct epitope recognition by Th cells that participate only in the induction of the serological response. Analogous data were obtained with the TgP1 sequence in intra-H-2 recombinant mice (Chapter 5). In studies with the human fibrinopeptide B in mice (L. B. Peterson *et al.*, 1983) it has been suggested that the fine specificities of T-cell responses to peptide antigens are different for helper and proliferating T cells. It has been postulated (T. R. Mossman & R. L. Coffman, 1989) that this may reflect recognition of distinct determinants by Th1 and Th2 cells. Clearly, further studies with TgP2-specific clonal T-cell populations will be needed to characterize the profile of Th cells involved in the generation of antibody and to distinguish them from Th cells responsible for the development of EAT.

CHAPTER 7

AUTOREACTIVE IgG ELICITED IN MICE BY THE NON-DOMINANT BUT PATHOGENIC THYROGLOBULIN PEPTIDE (2495-2511): IMPLICATIONS FOR THYROID AUTOIMMUNITY [1]

7.1 SUMMARY

We have previously shown that mice challenged with the RTg peptide TgP1 develop EAT and produce IgG antibodies that cross react with thyroglobulins from various species (Chapter 4). It was not clear, however, whether such antibodies were TgP1-specific or were induced secondarily, i.e. by autologous Tg released from the destroyed gland, and therefore directed to determinants other than TgP1. In this report we describe that, five weeks after priming with TgP1, the binding of serum IgG on native Tg is completely inhibited by free peptide, suggesting lack of recognition of other determinants on MTg. In addition, TgP1-induced but not MTg-induced IgG bound better to heat-denatured than intact MTg, a result compatible with the recognition of a linear epitope by the peptide induced antibodies. Comparison of the IgG subclass distribution among MTg-induced vs TgP1-induced IgG did not reveal qualitative differences, since all subclasses were represented in the order IgG1> IgG2b> IgG2a> IgG3. Finally, TgP1-specific IgG reacted strongly with the follicular colloid in sections of normal thyroids, indicating its potential to bind to native Tg *in vivo*. These data: 1) highlight TgP1 as the only, so far, Tg sequence known to generate both EAT and Tg-

[1] Part of the data presented in this chapter has been published as: Chronopoulou E., Michalak T. I. and Carayanniotis G. 1994. Autoreactive IgG elicited in mice by the non-dominant but pathogenic thyroglobulin peptide (2495-2511): Implications for thyroid autoimmunity. *Clin. exp. Immunol.*, Vol. 98: 89-94.

reactive IgG in mice and 2) do not provide evidence for an amplification of the Tg-specific IgG response through the involvement of endogenous autoantigen in EAT.

7.2 INTRODUCTION

In earlier work we have reported that the Tg peptide 2495-2511 (TgP1) encompasses non-dominant T-cell determinants and induces EAT in mice (Chapters 4, 5) and rats (B. Balasa & G. Carayanniotis, 1993b). This 17-mer peptide was selected for EAT induction because of its relatively high amphipathic score and the presence of amino-acid sequence motifs that characterize T-cell epitopes (Chapter 4). It was unexpected, therefore, to see that in mice, TgP1 elicited not only peptide-specific T cells but also IgG antibodies that bound to MTg. This finding was demonstrable despite the fact that mice challenged with MTg do not develop TgP1-specific IgG responses, strongly arguing against TgP1 being a B-cell epitope as well (Chapter 4).

An apparent correlation emerged, however, in that, following challenge with TgP1, the titer of MTg-reactive IgG was frequently high in mouse strains that developed EAT (Chapters 4, 5). This titer was usually lower or undetectable in mice which, after a similar challenge, exhibited no signs of lymphocytic infiltration of the thyroid. One interpretation we assigned to these data was that, following challenge with TgP1, EAT-susceptible-but not EAT-resistant strains elicited IgG reactive to a distinct TgP1 determinant which was accessible on MTg. An alternative interpretation, however, could be that the MTg-reactive IgG did not originate from TgP1-reactive B cells but was secondarily induced by self-Tg that entered the circulation as a result of thyroid damage. In the present study we have tested the latter hypothesis by comparing the reactivity

profile and subclass distribution of the MTg-specific IgG in the sera of susceptible mice challenged with either TgP1 or intact MTg.

7.3 RESULTS

7.3.1 In TgP1-primed mice, Tg-reactive IgG antibodies do not recognize determinants other than TgP1.

In a competitive ELISA, pooled sera (day 35) from TgP1-primed (see legend toTable 5.1) or MTg-primed (legend to table 4.2) B10.BR mice were allowed to bind to MTg in the presence of varying concentrations of free (soluble) TgP1. As shown in Figure 7.1, TgP1 concentrations higher than 0.22 μM completely inhibited IgG binding to MTg in sera from TgP1-primed animals. Inhibition was TgP1-dose-dependent and specific because it was not observed with another Tg peptide, TgP2 (a.a. 2695-2713) that causes EAT in mice (see Chapter 6) (data not shown). These results demonstrated that TgP1-specific IgG gained access to TgP1 on native Tg and was accountable for all the MTg-specific reactivity in the sera of TgP1-primed mice. On the other hand, the peptide did not inhibit IgG binding to MTg in sera of MTg-primed mice. This result confirms our earlier observations (see Chapter 4) which demonstrated that challenge with intact MTg does not elicit TgP1-specific IgG responses, thus defining TgP1 as a serologically non-dominant epitope.

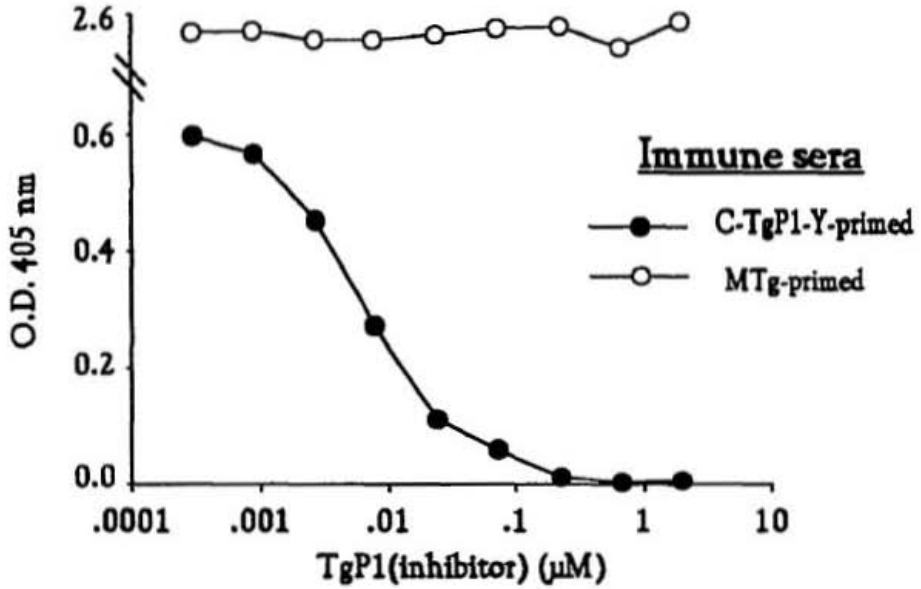


Figure 7.1: Competitive ELISA of serum IgG-binding to MTg using the 17-mer TgP1 as an inhibitor. Pooled sera (day 35) were obtained in earlier studies from B10.BR mice primed with either C-TgP1-Y (see table 5.1) or MTg (see table 4.2). Wells were coated with 1 μg MTg/100 μl. Each point represents the mean value of triplicate wells. Standard deviation did not exceed 5% of the means. Similar results were obtained in four replicate assays and with pooled sera from two experiments.

7.3.2 MTg-reactive IgG in TgP1-primed mice does not recognize conformational epitope(s).

If MTg-reactive IgG in TgP1-primed sera recognizes a linear epitope (TgP1), one would expect this recognition not to depend on the tertiary conformation of MTg. To test this, we utilized sera from C3H mice immunized with TgP1 or MTg (see legend to Table 4.2) in order to compare their binding on intact vs heat-denatured MTg. Heat-denaturation of MTg did not reduce the binding of TgP1-specific IgG to MTg, and in fact, slightly increased it (Fig. 7.2A) probably because the partial unfolding of the molecule allowed better access of this determinant to antibodies. It would be unlikely, therefore, that a more extensive denaturation of Tg (e.g. reduction-alkylation) would abrogate binding of TgP1- specific IgG. In contrast, MTg-primed sera known to contain IgG directed to conformational Tg determinants bound less well to denatured than intact Tg (Fig. 7.2B). These data provide no evidence for the presence of "secondary" IgG induced against conformational determinants of endogenous Tg during TgP1 challenge.

7.3.3 TgP1 and MTg elicit Tg-specific IgG with a similar subclass distribution pattern.

Because of its cryptic nature and short length, the pathogenic TgP1 sequence might stimulate the induction of Tg-reactive IgG that could significantly differ in terms of subclass distribution from the "conventional" IgG induced by intact MTg in EAT (see section 1.2.5; N. Fukuma et al., 1989). To ascertain this, day 35 sera of high responder B10.BR mice challenged either with TgP1 (see Table 5.1) or MTg (see Table 4.2) (7

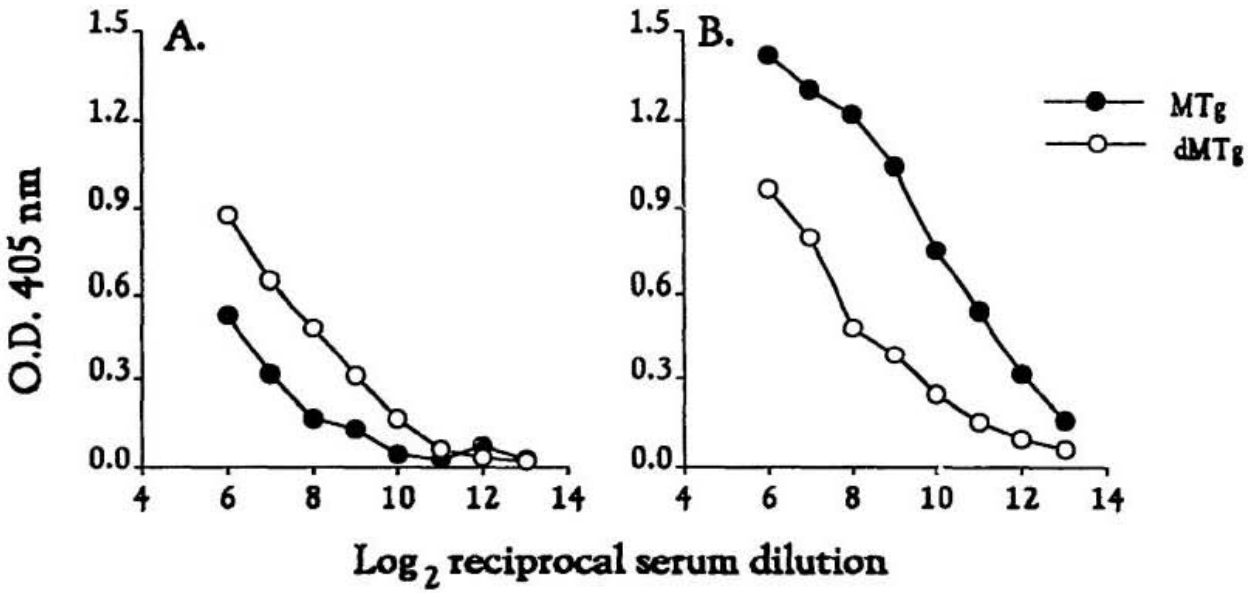


Figure 7.2: Heat-denaturation of MTg does not reduce the Tg-specific binding of serum IgG from mice primed with C-TgP1-Y. Pooled sera (day 35) from C3H mice, primed in earlier studies with C-TgP1-Y (Fig. 7.2A) or intact MTg (Fig. 7.2B), were allowed to react in an ELISA assay against intact (MTg), or heat-denatured (dMTg) thyroglobulin. Each point represents the mean value from triplicate wells. Standard deviations did not exceed 5% of the means. O. D. values are representative of data obtained in two replicate assays.

mice per group) were analyzed for the IgG subclass profile of Tg-reactive antibodies using an ELISA assay. As shown in Figure 7.3, all subclasses were represented in each group in the order IgG1> IgG2b> IgG2a>IgG3. However, TgP1-primed mice had, on average, a 10-50-fold lower concentration of Tg-reactive antibody (Fig. 7.3A) than mice primed with the intact antigen (Fig. 7.3B). This quantitative difference was demonstrable with all subclasses (IgG1: 9 vs 308 µg/ml serum; IgG2b: 1.2 vs 60 µg/ml serum; IgG2a: 0.2 vs 4.6 µg/ml serum; IgG3: 0.2 vs 2.8 µg/ml serum) and was expected since the intact self antigen can present a larger number of serological epitopes on its surface than TgP1. In addition, the observed quantitative differences were not strain-dependent because pooled TgP1-primed sera from SJL mice exhibited a similar pattern of subclass distribution as MTg-primed sera (Table 7.1). The data, however, did not provide evidence for restricted use of one or more IgG subclasses in the response to MTg after mouse challenge with TgP1.

7.3.4 Priming with TgP1 induces IgG that reacts with the follicular colloid of normal mouse thyroids

To confirm that Tg-specific IgG responses could be detected in an assay other than ELISA, we used fluorescence microscopy to examine the binding of Tg-reactive IgG on cryostat sections of normal SJL thyroids. After fixation with acetone, the sections were incubated with sera from TgP1-primed, TgP2-primed or normal SJL mice and then incubated with FITC-conjugated goat anti-mouse IgG. Sera from TgP1-primed mice reacted strongly to the follicular colloid (Fig. 7.4 A) whereas TgP2-primed or normal sera were non-reactive (Fig. 7.4 B, C). These results confirmed the ELISA observations and the notion that TgP1-induced IgG can react with this peptide

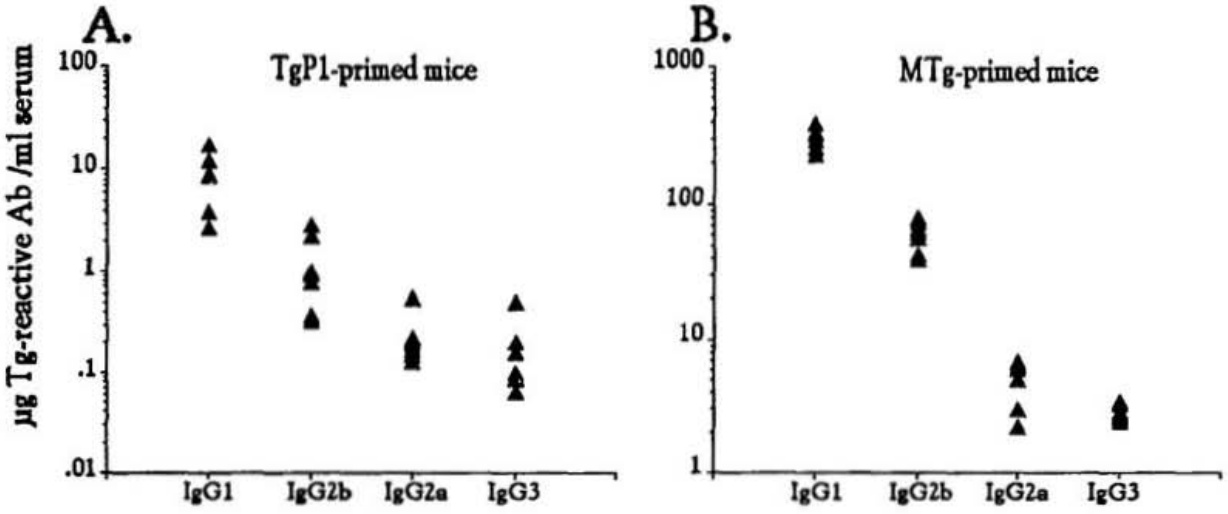


Figure 7.3: Subclass distribution of MTg-reactive IgG in the sera of B10.BR mice. Pooled sera derived from mice that were primed in earlier studies with TgP1 (Table 5.1) or MTg (Fig. 4.2) were tested for subclass distribution by ELISA (see *Materials and Methods*). Each point represents the value from a single mouse (7 mice per group). The values are extrapolated from a standard curve constructed as indicated in the *Materials and Methods*.

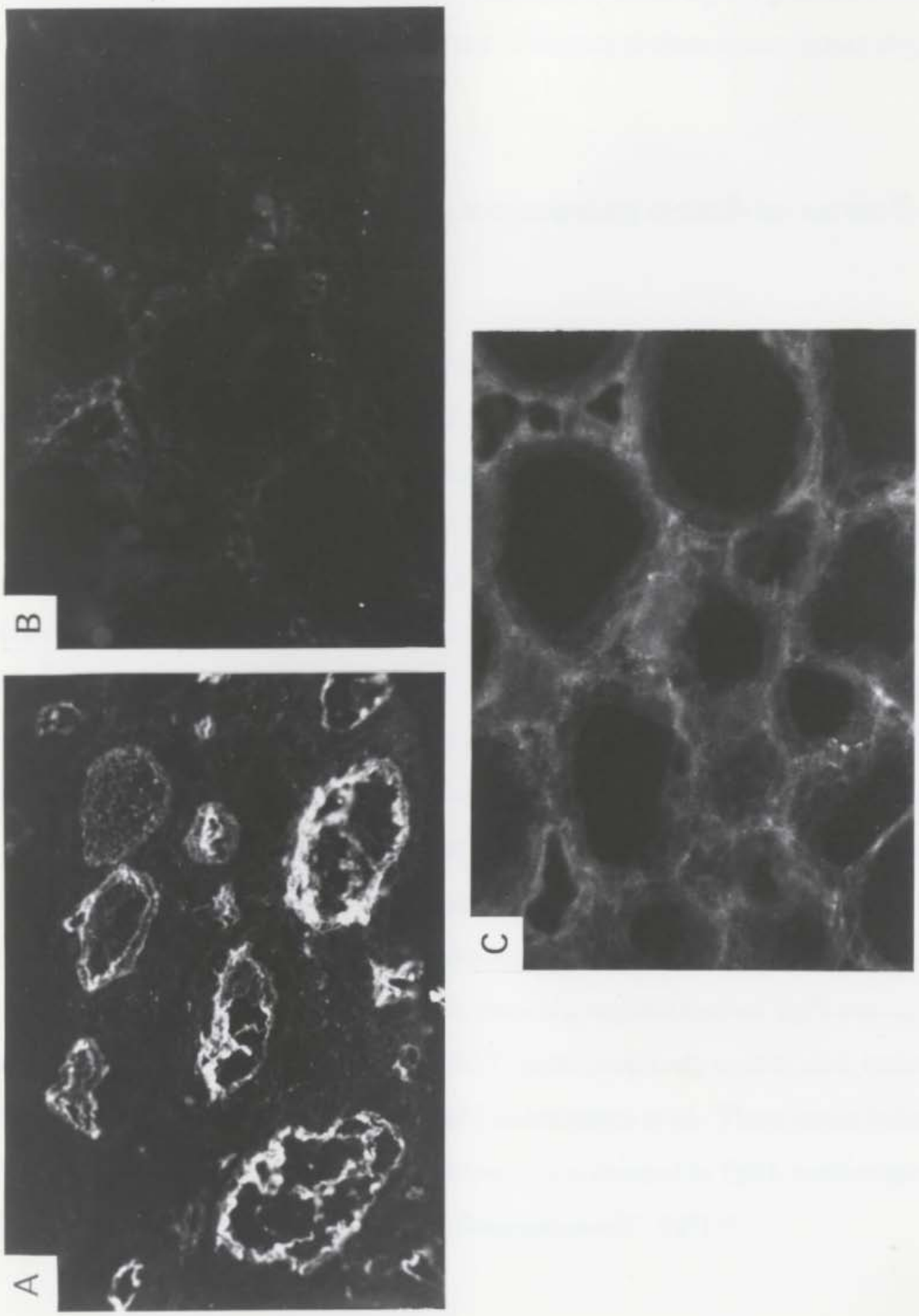
TABLE 7.1: Subclass distribution of Tg reactive IgG in different strains

Source of serum[a]	Ag *in vivo*	Antibody concentration (μg/ml)[b] IgG1	IgG2b	IgG2a	IgG3
B10.BR	TgP1	8.94	1.22	0.23	0.16
	MTg	308	60.3	4.62	2.82
SJL	TgP1	15.7	1.18	0.52	0.24
	MTg	652	53.56	10.34	4.11

[a] Pooled sera (7 mice /group) from B10.BR and SJL mice of fig. 4.2 were used.

[b] Values represent the means of triplicate wells.

Figure 7.4 A-C: Binding of SJL sera to sections of normal syngeneic thyroids as indicated by immunofluorescence. Binding pattern of immune sera following *in vivo* challenge with the 17-mer TgP1 (**A**), TgP2 (**B**). (**C**) Binding pattern of normal SJL serum (A, B, C X135).


A
B
C

sequence on intrathyroidal Tg. In addition, the lack of reactivity of TgP2-sera with the follicular colloid is compatible with the lack of binding of those sera to intact MTg in ELISA assays (Fig. 6.8A).

7.3.5 C-TgP1-Y dimerization affects its immunogenicity at the B- but not the T-cell level.

As noted in *Materials and Methods* in all of the *in vivo* and in most of the *in vitro* studies TgP1 carrying an amino-terminal cystein and a carboxy-terminal tyrosine (C-TgP1-Y) has been used. These two residues, foreign to the original sequence of amino acids, had been added for cross-linking and labelling purposes respectively. Sequences expressing amino-terminal cysteine, such as C-TgP1-Y, have the potential to create dimers that could affect the immunogenicity of the sequence at the B- and/or T- cell level. To exclude this possibility for C-TgP1-Y, we tested the ability of C-TgP1-Y-primed cells to proliferate *in vitro* to either TgP1 or C-TgP1-Y (Table 7.2). These data show that C-TgP1-Y-primed T cells can be equally well activated by both TgP1 and C-TgP1-Y sequences. Therefore, dimerization of C-TgP1-Y does not affect the epitope recognized by T cells on TgP1. To determine if the B-cell epitope is affected by dimerization, we attempted to inhibit the binding of sera from C-TgP1-Y-primed mice to C-TgP1-Y by either C-TgP1-Y or TgP1. In place of a negative control TgP2 was used as an inhibitor (Fig. 7.5). Although C-TgP1-Y could completely inhibit such binding, TgP1 produced a partial inhibition and TgP2 no inhibition at all. These results indicate that while a part of the C-TgP1-Y specific antibody is directed to TgP1, another part is directed to neo-determinants created after dimerization of C-TgP1-Y.

TABLE 7.2: Dimerization of C-TgP1-Y does not affect T-cell recognition of TgP1

Ag concentration *in vitro* (μg/ml)	Cell proliferation (mean cpm x 10^{-3}; S.I.) [a]	
	TgP1	C-TgP1-Y
10	23.8 (5.2) [b]	24.8 (5.5)
5	31.1 (6.8)	27.2 (6.0)
2.5	25.8 (5.6)	26.8 (5.9)
1.25	25.6 (5.6)	26.4 (5.8)
0.625	22.9 (5.0)	20.0 (4.4)

[a] C-TgP1-Y specific T-cell line (see legend to fig. 4.5) was tested for reactivity *in vitro* to either TgP1 or C-TgP1-Y. Proliferative responses were assessed by [^{3}H]TdR incorporation. Cpm in the absence of antigen was 4,577.

[b] Values represent the mean of an assay in triplicate. Values in parenthesis represent the stimulation indices.

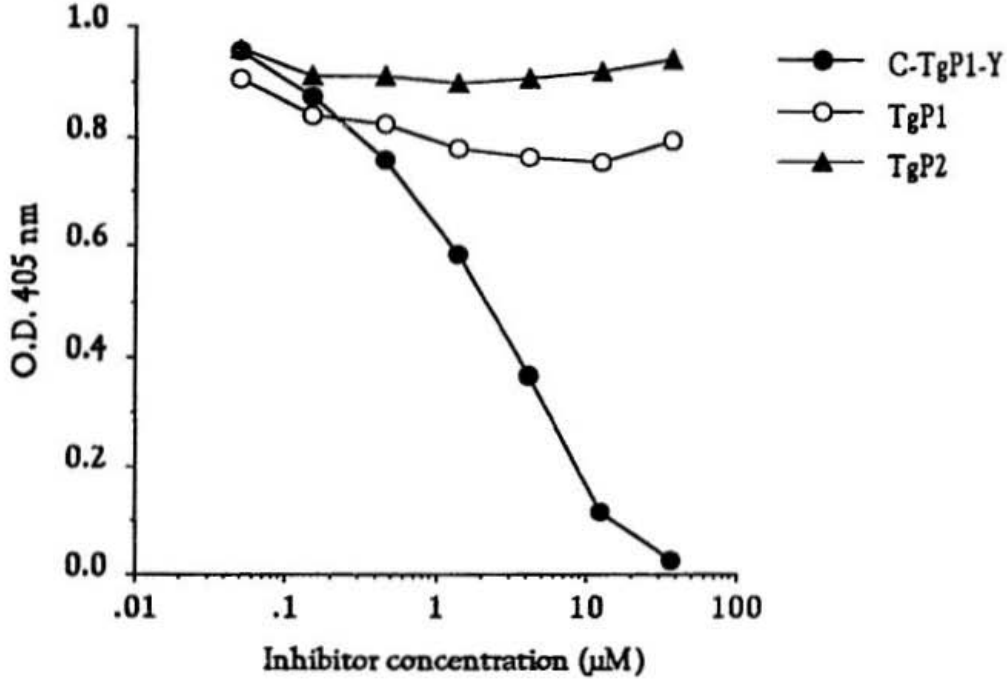


Figure 7.5: Competitive ELISA of serum IgG-binding to C-TgP1-Y by TgP1. Pooled sera from B10.BR mice of Table 5.1 immunized with C-TgP1-Y were inhibited from binding to C-TgP1-Y by free C-TgP1-Y, TgP1, and TgP2. Starting inhibitor concentration used was 36.64 μM. Serum dilution used was 1/2048. Each point represents the mean of an assay in triplicate.

7.3.6 C-TgP1-Y dimerization influences its coating efficiency during ELISA assays.

In all the ELISAs described in Chapters 4 and 5, 2 µg/ml of C-TgP1-Y had been used for microtiter-plate coating during the assays for C-TgP1-Y-specific antibody. In the current experiment, pooled sera (day 35) from various intra H-2 recombinant strains (Table 5.1) immunized with C-TgP1-Y were tested for binding to plates coated with 2 µg/ml of either TgP1 or C-TgP1-Y. Surprisingly, TgP1-primed serum bound strongly to C-TgP1-Y and less strongly to TgP1-coated plates (Fig. 7.6). To address whether the last result was simply due to the presence of additional antibodies within the C-TgP1-Y primed serum directed to neo-determinants (see previous paragraph) or to inefficient plate coating by TgP1, we set up the following assay. C-TgP1-Y primed sera from B10.A mice were tested for binding to plates coated with increasing amounts of TgP1. As shown in Fig. 7.7 the higher the concentration of TgP1 used, the better the binding of C-TgP1-Y primed serum to TgP1. The last result suggests that the coating efficiency of the two peptide forms varies.

7.4 DISCUSSION

EAT induction through the use of intact Tg in adjuvant implies initial recognition of dominant epitopes by T or B cells during the immunopathological process. We have recently shown, however, that, at least at the level of B cells, thyroid autoreactivity does not have to start from recognition of dominant determinants on Tg. EAT-susceptible strains such as B10.BR, C3H or SJL, challenged with intact MTg, do not elicit IgG that recognize TgP1 and yet TgP1 induces EAT and MTg-specific IgG in these animals. Since blood Tg levels, normally in the range of 60-120 ng/ml in mice (M. Lewis et al.,

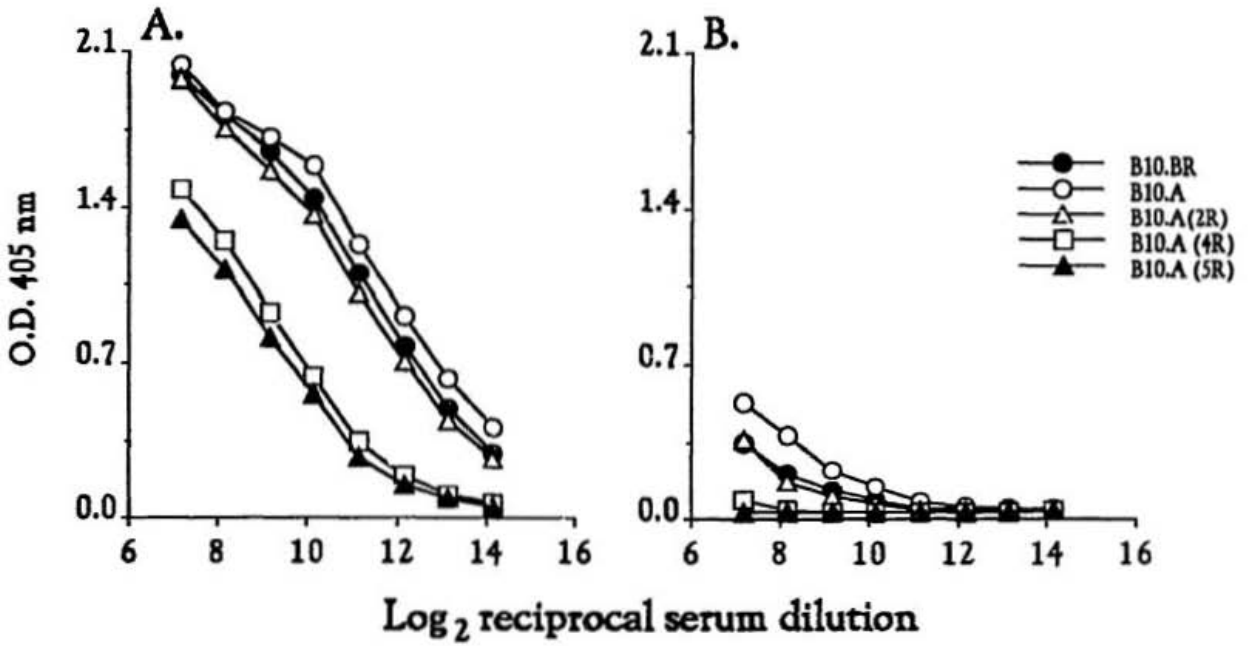


Figure 7.6: Determination of C-TgP1-Y-specific IgG binding to C-TgP1-Y and TgP1. Pooled sera from mice (7 animals /group) of Table 5.1 were tested for reactivity to C-TgP1-Y (A) and TgP1(B) by ELISA as described in *Materials and Methods*. Each point represents the mean value of triplicate wells.

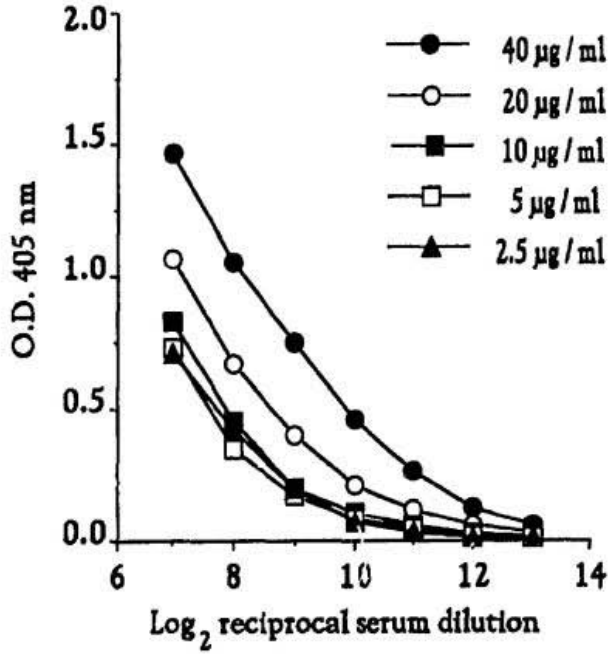


Figure 7.7: C-TgP1-Y-specific IgG binding to TgP1. Pooled sera from C-TgP1-Y-primed, B10.A mice (see legend to Table 5.1) were tested for binding to microtiter plates coated with various concentrations of TgP1. ELISA was performed as described in *Materials and Methods*.

1987), could increase during thyroid disease (A. J. Van Herle et al., 1979b) the possibility was raised that following TgP1-induced EAT, MTg-reactive IgG was elicited by endogenous Tg and directed to dominant Tg epitopes. The present data exclude this possibility and assign all the MTg-specific IgG reactivity to TgP1 specific IgG.

The present results also highlight TgP1 as the only, so far, Tg sequence known to elicit both EAT and Tg reactive IgG in mice. Other known pathogenic Tg peptides either do not behave similarly (see Chapter 6, 8; B. Texier et al., 1992b), or have not been tested for the above parameters (P. R. Hutchings et al., 1992). Autoreactive B cells might be triggered against non-dominant Tg peptides such as TgP1 by cross-reactive epitopes from bacteria (W. J. Penhale & P. R. Young, 1988) or viruses (J. Srinivasappa et al., 1988; T. Onodera & A. Awaya, 1990) linked to thyroid pathogenesis, although direct evidence for such events is still lacking. Once elicited, these autoantibodies may play a role in the initiation or amplification of the disease cascade via mechanisms such as ADCC (E. A. Calder et al., 1975) or immune complex deposition (J. A. Clagett et al., 1974) that have been suggested to participate in the disease process. It should be emphasized, however, that in TgP1-mediated EAT, the concomitant IgG response is not necessary for the development of thyroid lesions, since the disease can be adoptively transferred by peptide-specific T cells to naive recipients and it occurs in the absence of circulating autoantibody (V. P. Rao & G. Carayanniotis, unpublished observations). Similar data have also been obtained with TgP2 (see Chapter 6). Also, it remains to be seen whether transfer of serum containing TgP1-specific IgG is sufficient to induce EAT.

The earlier detection (Chapter 5) of TgP1 specific, non-MTg-reactive IgG in strains such as B10.A(4R) and B10.A(5R), that do not develop EAT after TgP1 challenge, can be partly attributed to recognition of neodeterminants, since the TgP1 peptide used in

initial studies carried an extraneous amino-terminal cystein and could be subject to dimerization. Supporting evidence for this claim is shown in Figure 7.5. It is clear, however, that the extraneous aminoterminal Cys and carboxyterminal Tyr residues in TgP1 are not required for immunogenicity since the native 17-mer peptide can induce a strong MTg-specific IgG response in mice, as shown by the reactivity of immune SJL sera to thyroid colloid (Fig. 7.4A) and by specific IgG binding to MTg in ELISA tests (Fig. 7.2). Similarly the two amino acids extraneous to the sequence affect neither T-cell reactivity (Table 7.2) nor pathogenicity because thyroiditogenicity has been mapped to nanomeric T-cell epitopes within the TgP1 sequence (V. P. Rao et al., 1994).

Five weeks after immunization, the subclass profile of the TgP1-specific IgG response in mice is qualitatively similar to the one elicited by homologous Tg, showing representation of all subclasses with a preponderance of IgG1 (mean values>80% of the total response) followed by IgG2b (mean values 10-20% of the total response). The data are in good agreement with previous findings of DeCarvalho and Roitt (1982) who described, by RIA, a similar IgG subclass distribution in the autoantibody response to homologous Tg in mice. Thus, the small molecular size and the non-dominant nature of the pathogenic TgP1 peptide, are not features that predispose induction of specific IgG with a limited subclass distribution. Our findings contrast with previous observations that have suggested that the antibody response to defined determinants of HTg is restricted to particular subclasses (N. Fukuma et al., 1989). The apparent contradiction may reflect differences in the time point that the subclass distribution was studied and/or the system studied (experimental vs spontaneous). Although it is possible that this subclass profile may change over time, evidence from HT patients indicates an essentially unchanged subclass distribution of Tg-specific IgG over 2 1/2-4 years (S. M. McLachlan et al., 1987).

Our data are in apparent contrast to the findings of Lou and Tung (1993) in autoimmune oophoritis, whc found that a T-cell peptide of the zona pellucida glycoprotein ZP3 elicited autoantibodies to ZP3 determinants localized outside the peptide (Y. Lou & K. S. K. Tung, 1993). This phenomenon was observed within 14 days following immunization, when oophoritis is established, but it was also detected as early as day 7 after challenge when oophoritis is not present. It was interpreted to involve endogenous ovarian antigen since it did not occur in ovariectomized mice immunized with the ZP3 peptide and this peptide did not contain B-cell epitopes that could cross-react with the native antigen. In our case, in EAT established 35 days after challenge with TgP1(Chapter 4) such "amplification" is not observed although it is possible that it might be detected at a later time. The differences in the experimental systems are too numerous to allow an assessment as to why TgP1 cannot act in analogous fashion in EAT. For example, the endogenous Tg levels may not rise sufficiently during TgP1-mediated EAT to facilitate induction of such a polyclonal B-cell response, or if they rise they may have suppressive effects (M. Lewis et al., 1987; M. Lewis et al., 1992). It is noteworthy that in experimental myasthenia gravis (T-M. Yeh & K. A. Krolick, 1990), rats immunized with a dominant T-cell peptide from acetylcholine receptor produce antibodies of broad specificities (multiple clonotypes) against the protein antigen only if they are subsequently challenged with acetylcholine receptor in saline. It remains to be seen whether injection of whole MTg in mice primed with the non-dominant TgP1 peptide can lead to an amplified anti-Tg antibody response.

A final point highlighted by our data is that the pathogenic TgP1 sequence is likely to be missed by methods employing mouse MoAbs for the identification of MTg-epitopes associated with thyroid disease (reviewed in R. C. Kuppers et al., 1991; see section 1.2.5). Such MoAbs are commonly derived using intact Tg as an immunogen

and thus allow recognition only of dominant epitopes on MTg. A similar partial assessment of determinants can be made on human Tg using heteroantisera induced by intact HTg. Indeed, several patients' sera with high anti-HTg titers do not inhibit the binding of murine MoAbs to HTg (R. C. Kuppers et al., 1991). In addition, sera of patients with autoimmune thyroiditis reactive with HTg may respond only to some (M. Henry et al., 1990) or no (Q. Dong et al., 1989) heteroepitope-bearing HTg fragments. In this regard, it is noteworthy that TgP1 does not overlap with any of these fragments (M. Henry et al., 1990; Q. Dong et al., 1989). Although the clinical relevance of TgP1 at both the T- or B-cell level remains to be established, our findings underline the importance of the synthetic peptide approach in the daunting task of mapping pathogenic Tg determinants. A combination of the above techniques with the availability of new algorithms and methodologies that ascertain MHC-bound epitopes may eventually allow a more rapid progress in this area.

CHAPTER 8

DISTINCTION BETWEEN IMMUNOGENICITY AND PATHOGENICITY IN THYROIDITIS. IDENTIFICATION OF AN IMMUNOGENIC Tg SEQUENCE THAT IS NOT PATHOGENIC.

8.1 SUMMARY

In the process of searching for Tg pathogenic T-cell epitopes through the algorithm approach, an 18-mer sequence, corresponding to a.a. 2500 to 2567 of HTg (TgP3), of the RTg molecule, was found to be immunogenic but not pathogenic in mice. Genetic analysis of the phenomenon using mice of various MHC haplotypes such as H-2^s, H-2^k, H-2^b and H-2^d, revealed a complete absence of thyroid abnormality. The peptide was clearly immunogenic in C3H, B10, B10.BR and BALB/c mice inducing TgP3-specific IgG. TgP3 failed to elicit antibodies in SJL mice. It is not known whether the lack of TgP3-specific IgG in SJL mice is due to absence of TgP3-specific B cells, T cells or to absence of both subsets. Sera derived from MTg-primed mice did not react significantly *in vitro* with TgP3 indicating that the peptide is not an immunodominant sequence at the B-cell level.

8.2 INTRODUCTION

In the last three years research has been focussed on the identification of Tg pathogenic T-cell epitopes aiming to use them for studying immunoregulatory mechanisms in thyroiditis (reviewed in K. Mignon-Godefroy *et al.*, 1994). In that

regard, four Tg pathogenic sequences recognized by autoreactive T-cells have been defined to induce EAT in mice (Chapters 4, 5; B. Texier *et al.*, 1992b; P. R. Hutchings, 1992). Among those pathogenic sequences the nanomeric Tg peptide 2551-2559 carrying T4 at position 2553 has been reported to generate effector cells and activate them *in vitro* to transfer EAT in normal recipients (P. R. Hutchings *et al.*, 1992).

In the current study, an 18-mer Tg sequence (TgP3) identified as a potential T-cell site by the "AMPHI" and "tetramer motif" algorithms has been tested for pathogenicity and antigenicity in various strains of mice. TgP3 encompasses the nanomeric pathogenic sequence reported previously but carries tyrosine instead of T4 at position 2553.

8.3 RESULTS

8.3.1 TgP3 encompasses several MHC-binding motifs.

TgP3 was selected as a potential T-cell epitope because it carries an amphipathic segment (positions 2550 to 2565) with high amphipathic score of 38.8 (block length, l=11) and two tetramer motifs starting at positions 2552 and 2558, respectively. TgP3 has also characteristics of I-E^k-binding peptides because it encompasses two hydrophobic residues, Phe2556 and Ser2557, seven residues apart from Arg2567 (J. A. Leighton *et al.*, 1991). In addition, it contains one sequence that follows the motif of DR-binding peptides (C. M. Hill *et al.*, 1991), since the bulky hydrophobic amino-acid Phe2556 is four residues apart from the small amino acid Glu2561. TgP3 encompasses a motif of naturally processed peptides bound to the HLA-DR4 allele (Phe2556-Thr2564), (R. M. Chicz *et al.*, 1993). A comparison of the counterpart to TgP3 sequences in other species reveals a great homology (Fig. 8.1). Its human homologue is identical to TgP3 whereas

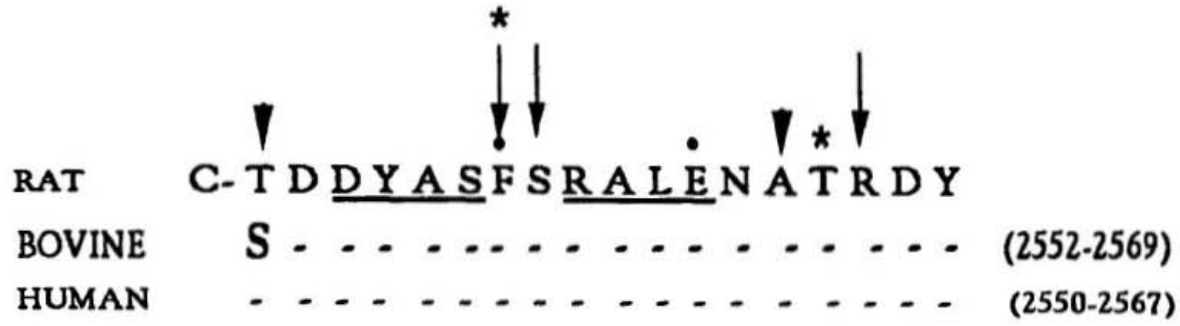


Figure 8.1: Primary amino-acid sequence of TgP3. *Underlines*, Rothbard and Taylor tetramer motifs; *short arrows*, amphipathic segment, according to the "AMPHI" program (amphipathic score=38.8, block length *l*=11); *long arrows*, a.a. in agreement with the I-E-binding motif of Leighton et al., (1991); •, a.a obeying the motif of Hill et al., (1991); *, a.a. delineate an HLA-DR4-binding motif (R. M. Chicz et al., 1993); -, a.a. identity between rat, bovine or human sequences at the positions shown. TgP3 carries an external to the sequence N-terminal cystein.

its bovine counterpart differs at a single amino-acid position from the rat sequence suggesting that TgP3 is a phylogenetically conserved Tg sequence. In addition TgP3 carries a hormonogenic tyrosine at position 2553 (see Chapter 1). Screening TgP3 with the PHYSCHEM program of PCGENE (see for details section 4.3.1) revealed other physicochemical features of TgP3 which are listed in Table 8.1.

8.3.2 TgP3 does not induce lymphocytic infiltration in the thyroid of H-2 $^{k, b,d,s}$ mice.

B10, B10.BR, C3H, SJL and BALB/c mice were primed s.c. at the base of the tail with 100 nmol of emulsified TgP3 in CFA and boosted three weeks later with 50 nmol of peptide in IFA. Control mice were similarly immunized with CFA/PBS. The thyroid glands of the immunized animals were examined histologically 3 weeks later for thyroid infiltration and scored as described in *Materials and Methods*. Under such conditions no thyroid infiltration was observed in any of the strains examined (Table 8.2; Fig. A, B). The thyroid architecture was similar to that obtained after immunization of the animals with CFA/PBS (Fig. 8.2 C, D).

8.3.3. TgP3 is immunogenic in H-2^{k,b,d} mice but not in H-2^{s} mice.

To address whether the lack of pathogenicity of TgP3 was due to lack of immunogenicity we measured TgP3-specific antibodies in the immunized animals. Pooled sera from the animals of Table 8.2 were tested for reactivity to TgP3 by ELISA (see *Materials and Methods*). TgP3-specific antibodies were demonstrated in B10,

TABLE 8.1: Physicochemical characteristics of TgP3 §

Molecular weight (MW)	2095
Isoelectric point (pI)	3.8
Half-life *in vitro*	
mammalian reticulocytes	7.2 hr
Half-life *in vivo*	
yeast	>20 hr
Escherichia coli	>10 hr

§ Physicochemical parameters of TgP3 according to the PHYSCHEM program of PCGENE (see section 4.3.1).

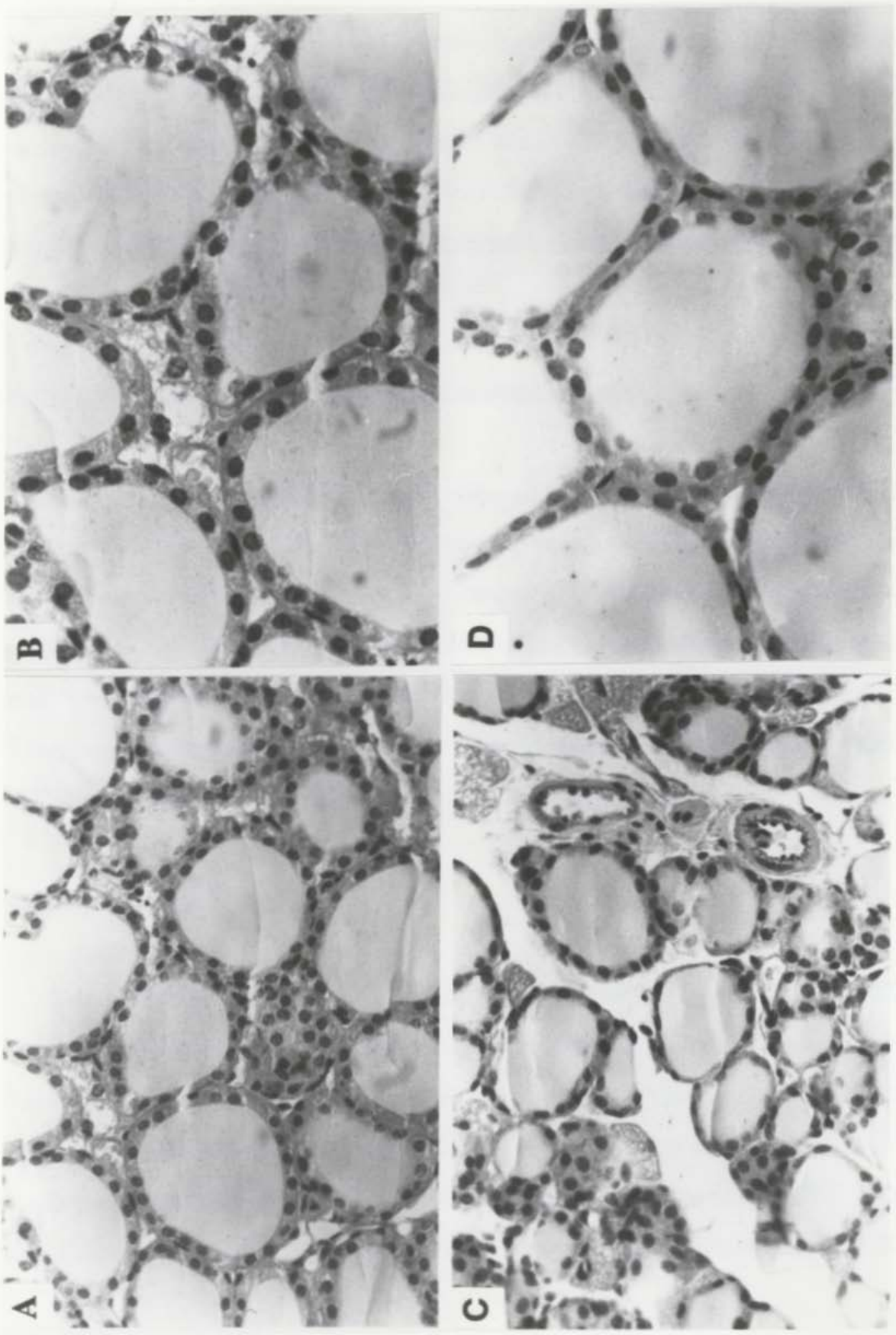
TABLE 8.2: EAT induction by TgP3 in various mouse strains.

Strain	H-2 haplotype	Infiltration index [a]						Mice with EAT
		0	0.5	1	2	3	4	
B10	b	4	0	0	0	0	0	0/4
B10.BR	k	4	0	0	0	0	0	0/4
SJL	s	4	0	0	0	0	0	0/4
BALB/c	d	4	0	0	0	0	0	0/4
C3H [b]	k	5	0	0	0	0	0	0/5

[a] Mice were challenged s.c. with 100 nmol TgP3 and three weeks later they were boosted s.c. with 50 nmol of peptide. Lymphocytic infiltration of the thyroid was assessed five weeks after the initial challenge as described in *Materials and Methods*.

[b] Mice came from a separate experiment. Immunization was performed as described in *a*.

Figure 8.2 A-D: Lack of abnormalities in the thyroid of C3H mice after TgP3 immunization. Thyroid sections were derived from animals immunized with either TgP3 A (X250), B (X400) or PBS/CFA, C (X250), D (X400).


B
D
A
C

B10.BR, C3H, and BALB/c mice suggesting that TgP3 is immunogenic in all of the above strains (Table 8.3). This last result provided indirect evidence that TgP3 is recognized by T cells. TgP3 failed to induce antibodies in SJL mice. Whether this lack of responsiveness to TgP3 is due to lack of T-cell help or to the absence of TgP3-specific B cells is unknown since, given the absence of pathogenicity, T-cell responses to TgP3 were not tested directly.

8.3.4 TgP3 is not a serologically immunodominant epitope.

Because TgP3 is recognized by B cells, although it had not been selected as a B-cell binding sequence, we attempted to determine if it was an immunodominant B-cell determinant. Pooled sera from the MTg-primed mice of Figure 4.2, were tested for reactivity to TgP3 by ELISA. Optical densities of less than 0.05 were observed in all the strains tested under conditions that produce strong reactions with MTg (Table 8.3). These results indicate that TgP3 is not a serologically immunodominant epitope. Finally TgP3-primed sera failed to react with MTg in ELISAs (Table 8.3).

8.4 DISCUSSION

Using the algorithm approach we could define an 18-mer TgP3 sequence within RTg that failed to induce thyroid lesions in a variety of mouse strains. Lack of pathogenicity, however, was not due to lack of immunogenicity in the strains tested because the peptide induced serological responses in the majority of those strains excluding SJL. Our findings are in agreement with recent results obtained by Hutchings *et al.*, (1992). Using the nanomeric sequence beginning at Asp2551 and carrying T4 at position 2553 (see

TABLE 8.3: TgP3 is not a serologically immunodominant epitope

Antigen *in vivo*[b]	Strain	O.D. (405 nm) of sera tested *in vitro* against[a]: MTg Ser. Dil. 1/128	MTg 1/512	TgP3 1/128	TgP3 1/512
MTg	B10	1.59	1.34	< 0.05	< 0.05
	B10.BR	1.65	1.48	< 0.05	< 0.05
	C3H[c]	1.39	1.19	< 0.05	< 0.05
	SJL	1.52	1.21	< 0.05	< 0.05
	BALB/c	1.24	0.96	< 0.05	< 0.05
TgP3	B10	< 0.05	< 0.05	1.22	0.98
	B10.BR	< 0.05	< 0.05	0.89	0.59
	C3H	< 0.05	< 0.05	0.85	0.37
	SJL	< 0.05	< 0.05	< 0.05	<0.05
	BALB/c	< 0.05	< 0.05	1.16	0.75

[a] Pooled sera from the mouse groups depicted in Table 8.2 were assessed for antigen-specific IgG by ELISA as described in *Materials and Methods*. The data were obtained from full titration curves and are expressed as means of triplicate wells at the indicated dilutions of serum. Standard deviations did not exceed 5% of the mean values.

[b] Mice were primed and boosted with MTg or TgP3 as described in *Materials and Methods*.

[c] Data come from a separate immunization.

Figure 6.1A) they were able to activate lymph node cells *in vitro* to transfer EAT to syngeneic animals. The presence of T4 was shown to be critical for the pathogenic potential of the sequence because replacement of T4 with cystein or other amino-acids abrogated its thyroiditogenic effects (B. R. Champion *et al.*, 1991; reviewed in K. Dawe *et al.*, 1993). Since this nanomeric sequence is included within TgP3, our study is consistent with the idea that iodination of that particular epitope is crucial for its pathogenicity. The underlying mechanism by which iodination influences disease pathogenicity in this case is possibly through formation of an immunodominant determinant that is recognized by autoreactive T cells (reviewed in R. S. Sundick *et al.*, 1992). It is worthwhile to mention, however, that in our study we attempted to induce EAT following the direct approach (see section 1.2.1.2). Following the same approach and using the nanomeric sequence at doses ranging from 1 to 25 μg per animal, Hutchings and colleagues failed to induce EAT in CBA/J mice. In the latter case lack of thyroiditis could be attributed to the low antigenic dose used for immunization of the animals. As shown in Chapters 4, 5, 6 an immunizing dose of 100 nmol which is approximately 200 μg of Tg peptide was required for thyroiditogenicity.

The present study shows that SJL mice are non-responsive to TgP3. Since we have not looked for T-cell responses in that strain we do not know whether unresponsiveness is due to lack of B or T cells recognizing the epitope or to the inability of the epitope to bind to class II molecules of H-2^s haplotype. Since TgP3 has been selected by two algorithms that identify T-cell sites on the basis of their intrinsic ability to form stable MHC-binding structures and do not take into account other factors such as the MHC alleles of the responding animal, the last explanation could be possible. For example TgP3 does not follow the motif found in natural peptides isolated and sequenced from I-A^s molecules (A. Y. Rudensky *et al.*, 1992).

TgP3 has been shown to induce strong serological responses in various strains, although presence of TgP3-specific antibodies did not correlate with thyroiditis induction. The last finding is compatible with results obtained by TgP1 (Chapters 4, 5), TgP2 (Chapter 6) and Tg (see section 1.2.3.3) and shows that antibodies cannot be used as markers of subsequent disease development. To explain the lack of pathogenicity of TgP3 in various strains several speculations can be made. First, TgP3 immunized animals may lack effector T cells. Second, they may lack Th cells that assist in thyroiditis induction. If that is the case, then the Th cells involved in the serological response versus those that participate in the disease pathogenicity, may be distinct. Third, TgP3 might not be expressed on the target organ as a product of Tg processing.

The present study demonstrates that TgP3 is serologically a non-dominant B cell-determinant. The non-immunodominant character of TgP3 is further supported by the study of Dong *et al.*, who failed to detect TgP3 among the 10 epitope-bearing fragments of HTg that are known to be recognized by heteroantisera (Q. Dong *et al.*, 1989).

CHAPTER 9

9.1 GENERAL OVERVIEW

Because T cells have been shown to play a crucial role in both induction and regulation of EAT (see section 1.2.3.1), an understanding of the fine mechanisms that operate in thyroid disease can only be achieved by focusing on this cell subset. One fundamental problem in that approach is to characterize those cells in terms of specificity. Such a characterization is crucial for the designing of specific immunointervention strategies. This problem can be tackled by two independent strategies. The first method relies on the isolation of the autoreactive T cells and the subsequent testing of their reactivity with antigenic fragments of the autoantigen. Alternatively, using defined T-cell epitopes, it is possible to isolate and characterize the autoreactive T cells. This study has been based on the second approach. Using as tools the AMPHI and "tetramer motif" algorithms, we were able to predict an array of sequences within rat Tg with structural characteristics of MHC-binding peptides. Among the candidate sequences, three were selected (see section 3.1) TgP1, TgP2, TgP3 and further tested in mice for both pathogenicity and immunogenicity. Based on the findings of this study the following conclusions can be drawn. First, the algorithm approach was successful in predicting T-cell sites but in several cases strain differences in the immune response to a given sequence were observed. Those differences were not unexpected because the algorithms used for the identification of the T-cell epitopes had not taken into consideration the allelic variability of MHC molecules that might influence the ability of the sequences to bind to the MHC. Moreover, various MHC alleles could influence the development of T-cells specific for a given self sequence in

the thymus by mechanisms of positive and negative selection. Second, although all three sequences were immunogenic in mice as revealed by their ability to elicit serological responses in various strains, only two of them (TgP1, TgP2) were pathogenic. This suggests that TgP1- and TgP2-specific antibodies cannot be used as diagnostic markers of mouse EAT and also that the Th cells that assist in the serological response versus those that help in the autoimmune attack of the thyroid might be distinct. Third, using defined T-cell epitopes to induce EAT, it is possible to investigate regulatory elements of the disease that are not recognizable in EAT induced by intact Tg. For example, TgP1-induced thyroiditis depends upon the control of I-E-region products whereas Tg-induced EAT is controlled by I-A-region products. Moreover, it was shown that TgP1-specific IgG which is elicited after immunization with TgP1 can bind to Tg stored in the follicular colloid of normal thyroids. This finding might imply a role for TgP1-specific IgG in EAT pathogenicity via mechanisms of ADCC or immune complex disposition. Further studies, however, are required to clarify such a role. Fifth, the pathogenic peptides TgP1 and TgP2 were characterized as non-dominant determinants at both the B- and T-cell levels. This last finding raises several questions. First, if the peptides constitute non-dominant determinants then how does EAT develop in mice? Second, what is the importance of cryptic epitopes in spontaneously-induced autoimmune thyroiditis in both animals and humans ? Although, we have not directly studied such questions in the current study we now have the tools to address them.

9.2 FUTURE DIRECTIONS.

This study provides an alternative, simplified model for studying immunoregulatory mechanisms in EAT (see section 1.2.4.1). Such immunoregularoty studies require, at an initial stage, generation of clonal T-cell populations specific for pathogenic epitopes of Tg and their subsequent characterization in terms of lymphokine secretion, MHC restriction, TCR and adhesion molecule expression, and function. This is an essential first step in understanding the cellular interactions and the costimulatory molecules involved in the activation of thyroiditogenic T cells. For example, evidence derived from the bulk cultures used in this study, suggests that TgP1 is recognized by both I-A- and I-E-restricted cells (see section 5.3.4). This has been confirmed by a recent study in which I-A- and I-E-restricted clonal populations have been isolated in the form of T-cell hybridomas (V. P. Rao *et al.*, 1994). With the aid of I-A- and I-E-restricted T-cell hybridomas and an array of truncated peptides of TgP1, it has been possible to define within TgP1 the minimal sequences that activate the hybridoma clones. The pathogenic and antigenic potential of I-A- and I-E-restricted epitopes has been then directly tested in thyroiditis susceptible mouse strains (V. P. Rao *et al.*, 1994). The identification of the minimal pathogenic sequences within TgP1 and TgP2 could be further used to address questions concerning the types of cells that are involved in EAT. By means of gene targeting technology it has been possible to disrupt genes of immunological importance such as the genes encoding interleukin-12 (IL-12), interleukin-4 (IL-4) (R. Kuhn *et al.*, 1991) and generate mutant strains of mice, "knock out" mice, that lack IL-12 and IL-4 respectively. Using such mice carrying the thyroiditis susceptibility haplotype and the minimal pathogenic epitopes of TgP1 mentioned previously, it is now feasible to analyze the importance of pathogenic TgP1-specific Th subset(s) in EAT pathogenesis by

immunizing IL-12 and IL-4 "knock out" mice with the I-A and I-E-restricted T-cell epitopes. An alternative approach that addresses the role of certain Th subsets and their secreted lymphokines in EAT, is to use the minimal epitopes as tools for the generation of I-A- and I-E-restricted T-cell clones. Such clones can be used for functional assays both *in vivo* and *in vitro*. For example, direct involvement of an individual Th subset in EAT induction can be revealed by adoptive transfer of an individual, well characterized T-cell clone to naive animals. Also, the involvement of a given T-cell clone in the antibody response can be determined *in vitro* by using it as a helper for purified TgP1-specific B cells.

Characterization of the MHC-restriction profile of the pathogenic Th clones and their TCR expression would facilitate the application of immunointervention approaches such as those described for the EAE model (see section 1.2.4.4). Previous studies have shown that Tg-induced EAT can be prevented in animals by "vaccination" with irradiated Tg-specific T-cell lines (R. Maron *et al.*, 1983) or a cytotoxic hybridoma clone that is specific for the epitope F40D (C. Roubaty *et al.*, 1990). In the latter case protection has been attributed to the production of anti-idiotypic antibody that reacts with the TCR of the cytotoxic T-cell clone (B. Texier *et al.*, 1992a). Analogous immunoregulatory studies can also be attempted with the non-dominant T-cell epitopes described in this study. Whether or not specific immunotherapeutic strategies such as the anti-Vβ therapy or anti-clonotypic vaccination can be employed in human thyroid disease in still a matter of debate (reviewed in C. M. Dayan *et al.*, 1992). The variables in the human system are too numerous to allow predictions. For example, in humans the T-cell specificities involved in the disease induction are still unknown, because the immunogen and the site of immunization have not be determined. In addition, experimental manipulations such as adoptive transfers that would reveal information

about the function of those cells cannot be employed in humans. Because the HLA alleles are highly polymorphic in humans, they allow binding of several peptides some of which might be pathogenic. Therefore, for one to employ successful immunotherapy in a particular individual, it might be necessary to suppress several pathogenic clones or to block more than one of the HLA alleles that are known to bind thyroiditogenic peptides. In that regard, to design specific immunotherapy at the population level it is necessary to identify the thyroiditis-associated HLA haplotypes, the pathogenic epitopes that bind to them and the specific T-cell receptors that recognize the epitope-HLA complexes.

In the current study, we have shown that the expression of H-2E molecules is a necessary but not sufficient requirement for EAT induction. It appears that other H-2 region products such as H-2K and/ or H-2A molecules are also involved in the disease process. The last conclusion is based on the finding that B10.A(4R) mice expressing H-$2K^k$ and H-$2A^k$ but not H-$2E^k$ molecules were completely resistant to thyroiditis induction. B10.A(5R) mice that express H-$2E^k$ molecules but H-$2A^b$ and H-$2K^b$ were also resistant to thyroiditis suggesting that H-2E expression is not sufficient for EAT induction (see Chapter 5). A recent study, however, does not favor the above conclusion (V. P. Rao *et al.*, 1994). In that study, the apparent resistance of B10.A(5R) mice to developing EAT, has been attributed to the expression of hybrid H-2E ($E\beta^b$:$E\alpha^k$) molecules which may not possibly be suitable heterodimers for TgP1 presentation. To determine whether or not resistance of B10.A(5R) animals is indeed due to the expression of a non-suitable E molecule and not to influences by H-2A and H-2K molecules, it is necessary to generate B10.A(5R) animals that express the $E\beta^k$ transgene. If a H-2E heterodimer derived from the susceptible haplotype is required, then $E\beta^k$-transgenic animals should develop thyroiditis. Alternatively, the same question can be addressed by generating B10 mice that express H-$2E^k$ molecules. Should these animals

develop EAT, this would indicate that TgP1-induced EAT is regulated solely by H-2E molecules.

TgP1 and TgP2 are non-dominant T-cell determinants which whould need,presumably, to be presented on the target organ in immunogenic form to be able to exert their pathogenic effect. To test this idea one could establish TEC cultures that originate from normal animals. TEC cultures that normally do not express class II can be transformed to class II^+ by IFN-γ (S. A. Ebner *et al.*, 1987) and can subsequently be used as APC for the activation of syngeneic TgP1 and TgP2 specific T-cell hybridoma clones in the absence of exogenously added peptide. This approach has been used previously for the immunodominant nanomeric pathogenic sequence that carries T4 (B. R. Champion *et al.*, 1991).

We do not yet know if TgP1 and TgP2 are important in spontaneously induced thyroiditis in either animals or humans or if, in fact, these pathogenic sequences are recognized by T cells of HT patients or by B and/or T cells of animals that spontaneously develop thyroiditis. Despite the lack of information in that direction, several speculations can be made. First, because the immune response directed to the peptides is MHC-dependent and the MHC genes are extremely polymorphic in humans, we expect to find variability in the responsiveness to those epitopes among individuals. Second, a sequence that appears as a cryptic epitope in an induced model of thyroiditis could be immunodominant in a spontaneous model. Immunodominance or crypticity must depend upon the antigenic factor that initiates the immunopathogenic process. In other words, although in the mouse model of Tg-induced EAT, TgP1 and TgP2 sequences are characterized as cryptic because other epitopes of Tg dominate the response, in spontaneously induced autoimmune thyroiditis TgP1 and TgP2 might be immunodominant. Such a concept can be easily tested using one of the available

animal models that develop SAT such as the NOD mouse or the OS chicken (see section 1.2.1.1). If TgP1, TgP2 are immunodominant epitopes at the B- and/or the T-cell level in a spontaneous model of autoimmune thyroiditis, then antibodies and/or T cells reactive to those epitopes should be found. Alternatively, the disease could start from a cryptic sequence, if that sequence happens to be recognized by cross-reactive T cells which are specific for an immunodominant determinant of a pathogen. In this context, it would be interesting to test whether or not human MHC can bind TgP1 and TgP2 and if human T cells specific for TgP1 and TgP2 exist in the thyroid infiltrate of patients with autoimmune thyroiditis. Preliminary studies in our laboratory indicate existence of TgP1 and TgP2-reactive IgG in patients with thyroid disease (G. Carayanniotis unpublished observations). However existence of TgP1 or TgP2 specific T cells has not yet been determined. We could also envisage that approaches such as those of computer-assisted modeling for MHC class II molecules would be valuable in predicting the binding of epitopes such as TgP1 and TgP2 to various MHC class II alleles (E. L. Huczko *et al.*, 1993).

In the past, several attempts have been made to detect T-cell reactivity to Tg in the peripheral blood of patients with thyroid disease (N. Shimojo *et al.*, 1988; N. Fukuma *et al.*, 1990). Those studies were somewhat inconclusive for two reasons (reviewed in C. M. Dayan *et al.*, 1992). First, T-cell reactivity to Tg in the periphery has been detected in healthy individuals as a part of the natural response. Second, since thyroiditis is not a systemic autoimmune disorder, the profile of T-cell reactivity in the periphery may not be representative of the situation in the target organ. Evidence supporting the latter point comes from studies in which only T-cell clones from the thyroid (M. Londei *et al.*, 1985) and not from the periphery (B. Grubeck-Loebenstein *et al.*, 1988) of Graves' disease patients could be activated by autologous TEC. It would be worthwhile to test if

clonal T-cell populations derived from the thyroids of patients with autoimmune thyroiditis can be activated *in vitro* by TgP1 or TgP2 and whether or not autologous TEC can stimulate such cells in the absence of exogenously supplemented peptide. A positive result would indicate a role for TgP1 and TgP2 in human disease.

It was formerly thought that the pathogenic epitopes on Tg must be limited. This notion was based on the observation that Tg-induced EAT is MHC-restricted (see section 1.2.2.1.1) and the conclusion that such a restriction profile could not be observed if a large number of thyroiditogenic determinants were included in the molecule. Our finding, do not favor this assumption because two of the three potential T-cell epitopes tested were shown to be pathogenic. In terms of immunogenicity, both pathogenic T-cell epitopes were characterized as non-dominant. This is a result that one could hardly get by chance. It seems likely that the huge Tg molecule harbors a large number of cryptic, potentially pathogenic, T-cell epitopes and a limited number of immunodominant, thyroiditogenic T-cell epitopes. In EAT induced by Tg immunization, the effects of non-dominant epitopes could be masked by those of the immunodominant T-cell epitopes towards which most of the autoimmune reactivity is directed. Cryptic epitopes, however, could be of importance in the evolution of Tg-induced EAT or in thyroiditis induced by infection or iodination. It would therefore be worthwhile to investigate, using immunodominant and non-dominant thyroiditogenic epitopes such as the nanomeric thyroxin-containing sequence (P. R. Hutchings *et al.*, 1992) and TgP1, TgP2 (this study), whether the spreading of autoimmunity from immunodominant to non-dominant T-cell epitopes, observed for EAE (P. V. Lehmann *et al.*, 1992; P. V. Lehmann *et al.*, 1993; K. D. Moudgil & E. E. Sercarz, 1994) is also found in EAT. If non-dominant epitopes are involved, then specific immunotherapy in autoimmunity might not be effective when directed to immunodominant epitopes of an

autoantigen only but might require that, for each haplotype, all the potential pathogenic epitopes be available.

It has been suggested that EAT induced by cryptic epitopes such as TgP1 and TgP2 might represent a model for the thyroiditis that develops spontaneously after infection or after increase of iodine uptake through the diet (see section 6.4). There are animal models in which thyroiditis induction has been attributed to pathogens such as the reovirus type I (S. Srinivasappa *et al.*, 1988), or to normal gastrointestinal flora (W. S. Penhale & P. R. Young, 1988). In these models, one might test whether T or B cells exist which are reactive to TgP1 and TgP2. This can similarly be tested in thyroiditis models where dietary iodine has been shown to influence the incidence and/ or severity of the disease (N. Bagchi *et al.*, 1985; E. M. Allen *et al.*, 1986). In summary, TgP1 and TgP2 are important tools for dissecting the mechanisms and cellular interactions of the autoimmune response in both animals and humans.

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APPENDIX 1

PREDICTED AMPHIPATHIC SEGMENTS

		MID POINTS OF BLOCKS	ANGLES	AS
	P	45-47	115.-130	6.4
		49-61	90.-130.	29.2
		102-109	110.-135.	17.7
*		133-135	105.-105.	5.6
*	P	138-142	80.-100.	10.0
	P	159-162	90.-100.	9.2
		194-195	110.-115.	4.6
*		207-214	125.-135.	18.1
		276-280	80.- 95.	9.1
		292-296	130.-135.	11.6
		309-310	135.-135.	4.9
*		317-321	125.-135.	8.0
		329-332	80.- 85.	5.7
		334-337	105.-115.	6.8
	P	394-397	120.-135.	7.1
	K P	404-432	90.-135.	67.4
*	K	445-455	115.-135.	23.9
	P	472-474	125.-135.	5.1
		524-526	100.-105.	7.9
		540-542	125.-135.	5.0
		548-550	90.- 95.	5.9
		553-563	110.-130.	21.8
	P	570-576	90.-105.	12.5
	K P	603-605	125.-130.	5.7
		635-636	95.-100.	4.2
*		646-651	85.-110.	13.2
	K	657-666	85.-110.	18.2
	P	672-684	80.-115.	24.2
	K P	687-700	80.-135.	23.9
		720-729	95.-120.	25.6
		740-745	80.- 95.	15.6
*		770-783	90.-120.	38.8
		796-797	105.-115.	4.7
	P	814-818	80.-115.	8.7
		823-828	100.-125.	12.2
		840-842	90.-100.	7.5
	P	856-867	95.-135.	28.1
	P	873-875	90.- 95.	6.9

P	881-890	85.-110.	23.3
P	894-904	80.-120.	24.1
K	918-934	95.-130.	39.6
	938-940	80.- 90.	4.9
K P	956-962	100.-105.	18.4

NO. OF PREDICTED BLOCKS 302

* indicates glycosylation site

P proline exists within the last 10 residues of the predicted segment

K is included at the N terminus of the predicted segment

APPENDIX 2

Prediction of "tetramer motif" sequences within the rat Tg molecule

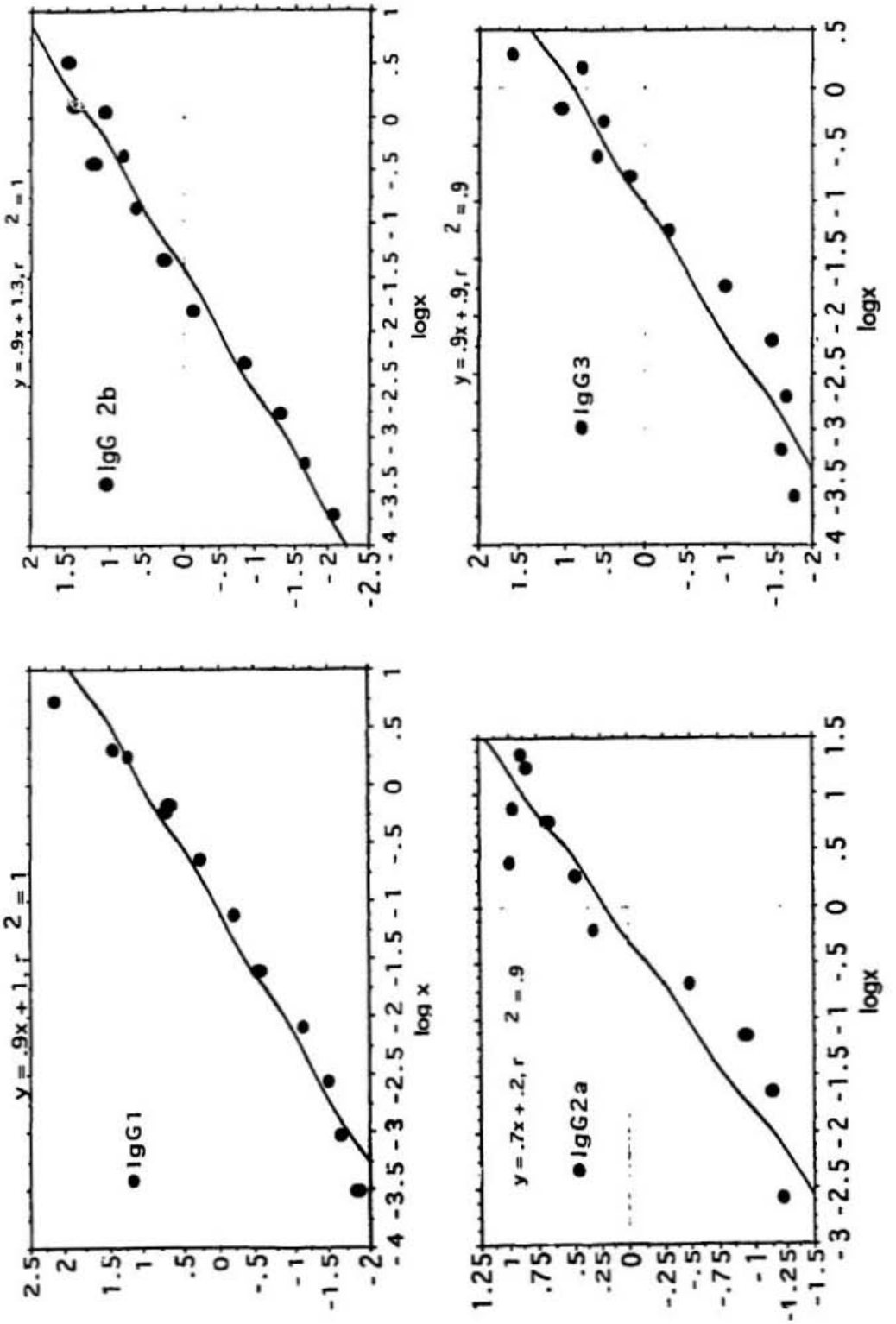
Sequence	a.a. position within RTg
EAPE	42-45
DMAT	48-51
ELFS	52-55
DITQ	58-61
HLFS	80-83
DIME	106-109
KLIS	185-188
GFFE	190-193
GFLN	208-211
GWYQ	259-262
DAPS	269-272
DVAH	310-313
GVVK	349-352
EVLN	441-450
DATK	456-459
RTPT	470-473
GVFG	538-541
EVAG	549-552
GLLD	555-558
GAFG	571-574
DVAS	590-593
EVVS	649-652
HYWG	677-680
ELPS	690-693
GLIN	715-718
KAVK	721-724
DYAS	772-775
RALE	778-781
HYPE	812-815
GYKS	842-845
KVMQ	854-857
KAAE	877-880
KYIQ	920-923

Appendix 3: Linear transformation of standard curves for IgG subclasses. Standard curves for individual IgG subclasses were established as described in *Materials and Methods* (data not shown). The s-shaped subclass titration curves were subsequently converted to straight lines according to the Von Krogh equation:

$$\log x = \log k + 1 / n \log y / 1-y,$$

where y is the O.D. of a subclass at a certain serum dilution expressed as a percentage of the maximum O.D. obtained for that subclass and x is the antibody concentration expressed in mg/ml of solution.

APPENDIX 3


y = .9x + 1.3, r 2 = 1
IgG 2b
logx
y = .9x + .9, r 2 = .9
IgG3
logx
y = .9x + 1, r 2 = 1
IgG1
log x
y = .7x + .2, r 2 = .9
IgG2a
logx