T-cell recognition in experimental autoimmune thyroiditis

by

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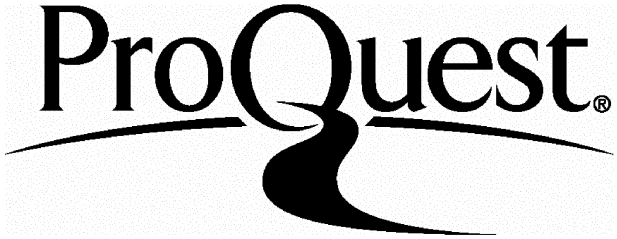

Abstract.

A pathogenic thyroxine containing epitope involved in the induction of experimental autoimmune thyroiditis has been mapped to the C-terminal region of human thyroglobulin. Using thyroglobulin reactive T-cell hybridomas we have been able to define the minimal epitope required for class II restricted recognition, this has been shown to be 9 amino acids in length. Both the iodination and the structure of the thyroxine residue have been shown to be critical for T-cell recognition by thyroglobulin reactive T-cell hybridomas and for subsequent activation. This recognition can be inhibited by the use of antibodies directed to the thyroxine residue of the peptide thus giving us useful clues about the possible three-dimensional structure of the class II-peptide complex. Potential MHC/TCR binding residues have been identified by the extensive use of substituted peptides we have been able to demonstrate two residues whose substitution abrogates peptide activity, it is not known if these residues bind MHC or TCR.

The identified epitope is able to re-activate MTg primed lymph-node cells *in vitro* and *in vivo;* such that they are able to transfer disease to naive recipients, however cells primed with thyroglobulin depleted of iodine are unable to recognise thyroxine containing peptide *in vitro.* Anti-CD4 therapy *in vivo* following MTg priming is able to specifically down-regulate *in vivo* and *in vitro* responses to thyroxine containing peptide. Using a selection of susceptible mouse haplotypes some identification has been made regarding other thyroxine containing residues that may be involved in the induction of experimental disease. The DBA/1 haplotype appears to respond to a thyroxine containing peptide covering position 5 on the thyroglobulin molecule. This is in contrast to the CBA/J which responds to the peptide covering position 2553 following priming with MTg and the SJL which does not seems to recognise thyroxine containing epitopes.

The thyroxine containing peptide covering position 2553 can be presented by thyroid epithelial cells in the presence of class II to T-cell hybridomas recognising this region. However, under similar physiological conditions, pathogenic lymph node cells seem unable to recognise these peptide/class II complexes. It has been shown that thyrocytes from high responder strains are susceptible to class II induction by IFN-γ which can act synergistically with TNF-α to up-regulate levels. Although it appears that levels of class II induced may not differ significantly between high and low responder strains, this implies that there are other factors besides MHC association that may be important in the presentation by thyroid epithelial cells.

Acknowledgements.

I wish to express my eternal gratitude to Mrs Paddy Hutchings for her sound advice, confidance, intellectual discussion and scientific collaboration. Most of all I want to thank her for her friendship, without any of these things life would have been very boring!

I would also like to thank Professor Ivan Roitt and Dr Anne Cooke for their help and encouragement they have given me in recent years and for reading this thesis. I would also like to thank Professor Ivan Roitt for giving me the opportunity to work in his laboratory; it has been a priviledge and a pleasure to know him. Many thanks also go to Dr Brian Champion for his help and advice.

Most of the thanks must go to my husband, Mark. Without his stubborn persistence and nagging; but more importantly his help and love, I could not have written this thesis and remained sane at the same time. For those things and many many others I will be forever grateful, this thesis is for him as well as myself.

Kim Isabelle Hickery (nee Dawe).

Dedication.

" Everything we are is that way because that was how our parents made us. Every talent we have has been inherited. And this is something worth remembering if we ever feel ourselves getting a bit swanky. The credit is not ours: it is theirs. Not even theirs really, but their parents."

A.A. Milne. 1931.

For my Parents and Grandparents, I could not have done this without you.

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Abbreviations.

aa	amino acid
AAb	Autoantibody
ADCC	Antibody-dependent cell mediated cytotoxicity
APC	Antigen presenting cells
ATA	3-amino-1,2,4-triazole
ATP	Adenosine triphosphate
bp	Base pairs
Bx	Bursectomy
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CFA	Complete Freund's adjuvant
CG	Choriogonadotrophin
CNS	Central nervous system
ConA	Concanavalin A
CS	Cornell strain
CsA	Cyclosporin A
Cy	Cyclophosphamide
DIT	Di-iodthyronine
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EAT	Experimental autoimmune thyroiditis
EBV	Epstein Barr virus
ELAM-1	Endothelial adhesion molecule-1
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FT4	Free T4
GMCSF	Granulocyte monocyte colony stimulating factor
HEV	High endothelial venules
HLA	Human leucocyte antigen
HTg	Human thyroglobulin
i.p.	Intraperitoneal
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IL-2	Interleukin-2

IL-4	Interleukin-4
IL-10	Interleukin-10
kb	Kilobases
kDa	Kilodaltons
LFA-1	Lymphocyte function associated antigen-1
LH	Luteinising hormone
LNC	Lymph node cells
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MBP	Myelin basic protein
MHC	Major histocompatibility complex.
MIT	Mono-iodothyronine
mRNA	messenger RNA
MTg	Mouse thyroglobulin
NK	Natural killer
NTx	Neonatal thymectomy
OS	Obese strain
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PIP_2	Phosphatidyl inositol 4,5-bisphosphate
PIP_3	Phosphatidyl inositol 1,4,5-triphosphate
PKC	Protein kinase C
PPD	Purified protein derivative
PWM	Pokeweed mitogen
r-mu-	Recombinant murine
RBC	Red blood cells
RER	Rough endoplasmic reticulum
r-h-	Recombinant human
RT	Room temperature
rT3	Reverse T3
SAS	Saturated ammonium sulphate
SAT	Spontaneous autoimmune thyroiditis
SRBC	Sheep red blood cells
T3	Tri-iodothyronine
T4	Thyroxine
TCC	Terminal complement complex
TCR	T-cell receptor

TEC	Thyroid epithelial cell
Tg	Thyroglobulin
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
TPO	Thyroperoxidase
TRH	Thyroid releasing hormone
Tc	Cytotoxic T-cell
Th	Helper T-cell
Ts	Suppressor T-cell
TSab	Thyroid stimulating antibody
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
TTF1	Thyroid transcription factor-1
Tx	Thymectomy
VCAM-1	Vascular adhesion molecule-1

1.0 Introduction.

1.1 Physiology of the thyroid.

1.1.1 Macroscopic structure.

The thyroid is a bi-lobed organ connected by an isthmus which crosses in front of the trachea at the second to fourth cartilage rings and is enclosed by connective tissue that is continuous with the peritrachial fasia. The outer capsule is loosely connected to a deeper layer of tissue that forms the inner capsule and the region between the two capsules containing the nerve supply and the parathyroid glands. An ample blood supply is provided by the superior and inferior thyroid arteries which are derived from the carotid artery, these arteries ramify on the surface of the gland forming a plexus with branches that enter the tissue. These branches eventually form a network of capillaries which encapsulate the follicles, the capillaries are fenestrated and run close to the follicles such that there is no intervening tissue. The veins also form a plexus on the surface of the gland which is drained by the superior, middle and inferior thyroid veins. These veins drain into the lateral jugular vein whilst the inferior opens into the brachio-cephalic vein. Lymphocytes are often found in close association with the thyroid follicles; most occur in the interlobular connective tissue surrounding the arteries and communicate with the lymphatics in the capsules. The drainage to the lymphatic system is to the nearby nodes located on the thyroid, trachea and larynx and also to the cervical nodes along the jugular vein. Along with an ample blood supply, the thyroid also receives sympathetic and parasympathetic innervation; nerve fibres are seen to terminate close to the thyroid follicles and the capillaries.

1.1.2 Microscopic structure.

A) Follicular structure.

From the inner capsule, septa extend into the gland and divide it up into many lobules which themselves are composed of follicles. A follicle consists of a single layer of epithelial cells which enclose the follicular lumen, which itself contains the colloid. The follicle is surrounded by a thin basement lamina which consists of a network of reticular fibres, capillaries and nerve fibres. Thyroid cell shape varies between species and within follicles; in the human the cells are usually squamous cuboidal whereas in the rat they are low cuboidal to low columnar. Although the follicle relies on its constituent components for the production of the thyroid hormones, it is the follicle itself which is the functional unit of the thyroid. It gives the gland not only the all important characteristics of production and storage of hormones but also the characteristic of immediate supply on demand.

Within the thyroid cell, the dominant organelle is the rough endoplasmic reticulum which emphasises the important speciality of the cell to synthesise thyroglobulin (Tg). This is a

protein specifically synthesized by the thyroid gland for the production of the thyroid hormones, thyroxine and tri-iodothyronine. The cisternae within the thyroid cell are wide and contain a low to moderate density granular material which is Tg; it is now known that the majority of the synthetic capacity of the RER is directed towards Tg synthesis. Following gene translation, synthesis of Tg takes place in polysomes attached to the RER membrane (Vassart *et al.*, 1973). Before exiting the RER, the leader sequence is cleaved and the thyroglobulin molecule folded and dimerised (Kim & Arvan, 1991). Tg is then transferred to the Golgi body via transitory vesicles and it is here that most of the peripheral monosaccharides are incorporated onto the carbohydrate units of Tg. Thyroglobulin is then transferred to the apical cell surface enclosed in vesicles (Bjorkman *et al.*, 1974, Ekholm *et al.*, 1975a), the contents of which are emptied into the follicular lumen at the apical surface by the process of exocytosis. Thyroglobulin plays an essential role in thyroid hormone homeostasis in that it allows the formation of hormones in a pro-form (inactive) which can be stored at length in an extracellular space free from metabolic influences. The thyroid is able to scavenge iodine from the diet effectively and as such the normal human thyroid contains enough hormone to sustain a euthyroid state for two months in the absence of any new synthesis.

B) Production of thyroglobulin.

Thyroglobulin gene structure.

The human Tg gene is a single copy gene with no closely related pseudogenes (Van Ommen *et al.*, 1983) and is located on chromosome eight in the human (Van Ommen *et al.*, 1984). It is located distal to the c-myc oncogene in humans and the rat (Baas *et al.*, 1984, Brocas *et al.*, 1984) which has been implicated in some thyroid tumours expressing high levels of Tg. The gene itself is 300 kb long and is a split gene in which there is a predominance of small exons of 150-200 bp long. Their distribution is homogenous throughout the length of the gene and there are two exons of 500 and 1000 bp towards the 5' end (Christophe *et al.*, 1982, Avvedimento *et al.*, 1984). Using cDNA clones, the structures of bovine (Mercken *et al.*, 1985), rat (Musti *et al.*, 1986) and human Tg (Malthiery *et al.*, 1987) have now been deduced. Previous studies on thyroglobulin have suggested that Tg contains well defined sites for hormone synthesis in which hormonogenic residues are located in a structural environment appropriate for iodination and coupling. There is strong homology in the primary sequence surrounding the thyroxine forming sites in thyroglobulin suggesting that there is a strong requirement for conservation of the three-dimensional structure at these points.

In the human, an 8448 nucleotide mRNA encodes a 2748 amino acid protein (Malthiey *et al.*, 1987) whereas the bovine mRNA (8434 nucleotides) encodes a 2750 amino acid sequence. It is believed that the Tg gene may have arisen by gene duplication both the 3' and 5' ends of the gene are made up of repeating units but these ends do not share homology.

It is suggested that both regions arose by serial duplication of different ancestral sequences which evolved independently towards the common function of encoding a thyroxine forming site (Musti *et al.*, 1986). The N-terminal region of thyroglobulin molecule shares a repeat homology with the invariant chain (Koch *et al.*, 1987) and there is a 600 residue region at the C-terminus which shares homology to acetylcholinesterase and other esterases (Malthiery *et al.*, 1985, Takagi *et al.*, 1991).

The first hormonogenic tyrosine is located towards the N-terminal at position 5 (Lejeune *et al.*, 1983a), which is preceded by a 19aa leader hydrophobic sequence and is involved in Tg secretion (Marriq *et al.*, 1984). Situated within the Tg sequence there are also 20 signal tri-peptides (ASN-X-THR/SER) which are potential N-linked glycosylation sites. The N-terminal tyrosine residue at position 5 is a site for thyroxine (T4) formation, in that this is the hormone is most commonly found at this site (Malthiery *et al.*, 1985, Lejeune *et al.*, 1983b). Under conditions of normal iodine intake this site is responsible for the synthesis of nearly 50% of the total T4 produced and there has been shown to be a high degree of conservation of the four hormonogenic acceptor sites especially that at position 5 (Rawitch *et al.*, 1984, Mercken *et al.*, 1982). At minimal levels of iodination it has been shown that this residue is the first to be iodinated in the human Tg molecule (Plaumbo *et al.*, 1990). There are two hormonogenic sites located approximately 200aa from the C-terminus at positions 2553 and 2567 in human Tg (Malthiery *et al.*, 1985) which have also been identified as hormonogenic sites in porcine Tg (Marriq *et al.*, 1982) and bovine Tg (Mercken *et al.*, 1985); a different site has been located on rabbit Tg which is residue 1291 (Dunn *et al.*, 1987). The final hormonogenic site is located just 5'of the C-terminus and has been shown to be a unique tri-iodothyronine (T3) forming site in porcine (Marriq *et al.*, 1983) and bovine Tg (Mercken *et al.*, 1984). Thus the major T4 forming site and T3 forming site are located at the opposite ends of the molecule. It is not known whether this is because these sites are more accessible to iodination and therefore favours iodotyrosine coupling. Their situation may also favour protease enzymatic attack thus permitting easier release and therefore secretion of thyroid hormones. In general the C-terminal region appears to contain a higher proportion of tyrosine residues than the rest of the Tg polypeptide chain. The position of the hormonogenic sites in human Tg are shown in figure 1.1 and the amino acid sequences surrounding these sites are shown in figure1. 2.

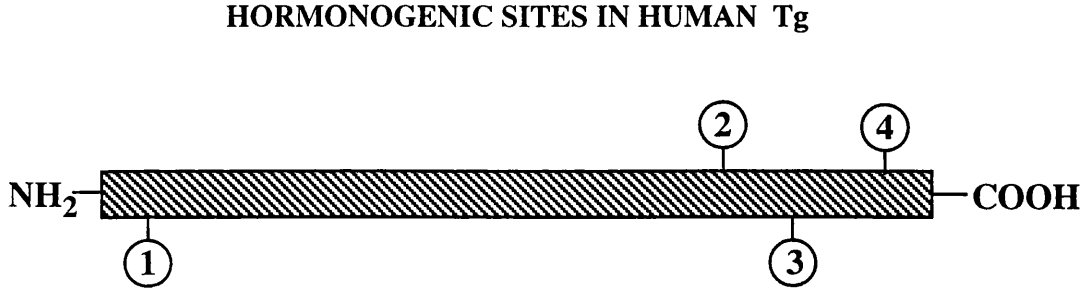
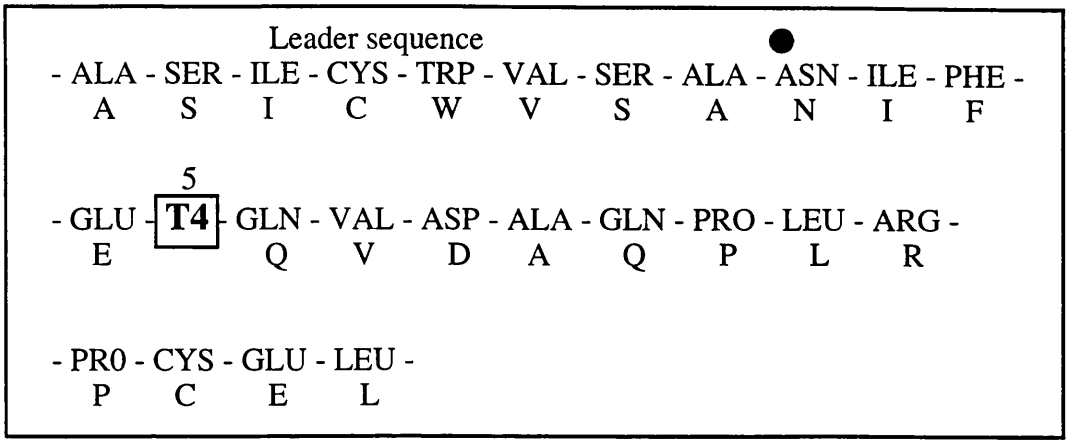
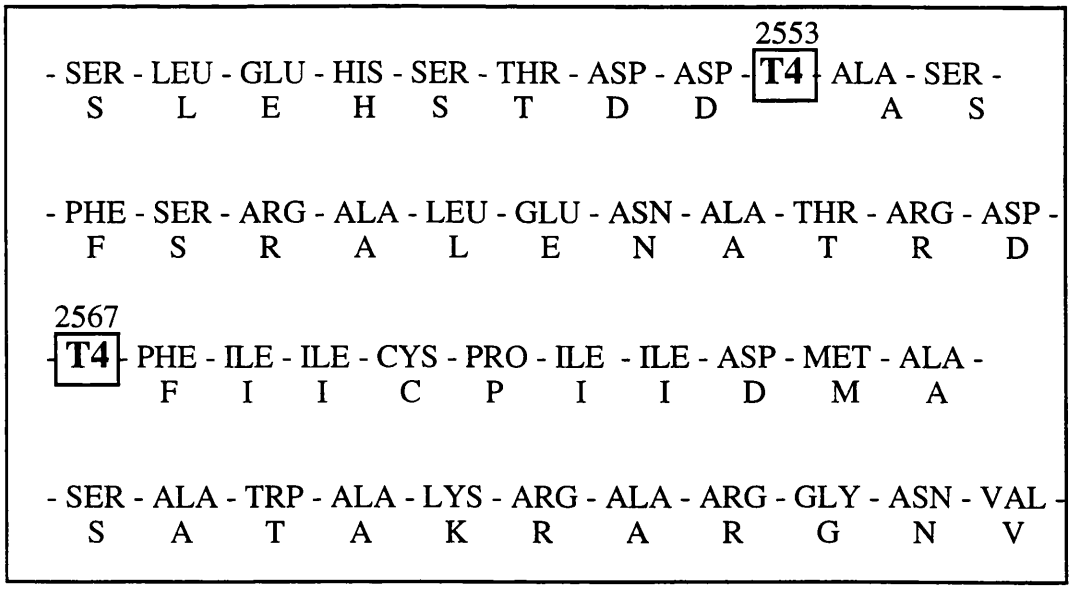


Figure 1.1. Position of the four hormonogenic sites in human Tg.

T4 residue 5.



T4 residues 2553 and 2567.



T4 residue 2746.

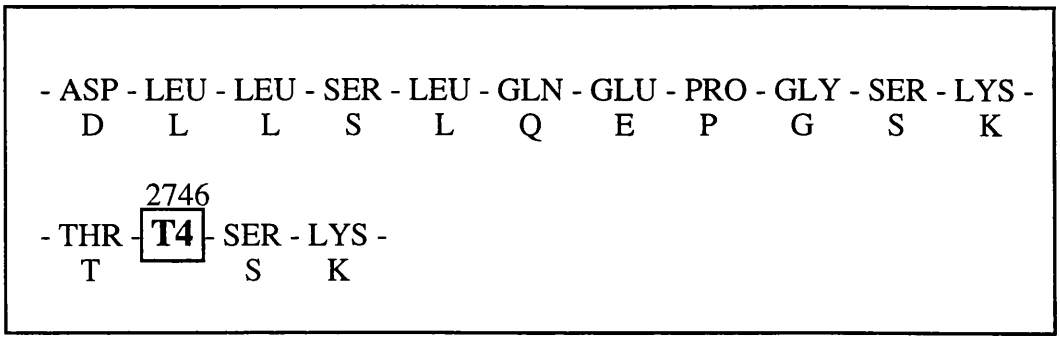


Figure 1.2. Amino acid sequences surrounding the T4 sites in human Tg.

Thyroglobulin biosynthesis.

Thyroglobulin is glycosylated at scattered points along the polypeptide chain (Malthiery & Lissitzky, 1987) and comprises 8-19% by weight of the whole protein, depending on species (Tsuji *et al.*, 1981,Yamamoto *et al.*, 1981). After synthesis, Tg chains are released into the cisternae of the ER by a mechanism involving the signal peptide located in the 5' region of Tg mRNA (Malthiery *et al.*, 1985, Marriq *et al.*, 1984). Oligosaccharides involved in the glycosylation of Tg are of the generalised structure $(GlcNAc)_2(Man)_n(Glc)_n$ and these are pre-assembled in the ER (Ronin *et al.*, 1978). These oligosaccharides are attached along the polypeptide chain at signal sequences ASN-X-SER/THR, the reaction is catalysed by an oligosaccharide transferase. Glycosylation is then completed in the Golgi, the entire process of the synthesis of the polypeptide chains and their glycosylation takes approximately 3h.

Thyroglobulin iodination and formation of the hormonogenic sites.

The iodination of thyroglobulin is a crucial step in the formation of thyroid hormones since thyroxine (T4) and tri-iodothyronine (T3) are required in an iodinated form for function. Iodination of thyroglobulin takes place at the apical membrane (Ekholm *et al.*, 1975b, Ekholm *et al.*, 1981) prior to storage in the follicular lumen. Iodide is transported into the cell via the iodide pump; it is present in the plasma as a very dilute constituent but iodide transport results in a concentration gradient from plasma to cell of 20-40 fold. This active transport mechanism is temperature dependent and requires ATP and is therefore inhibited by inhibitors of aerobic metabolism. The pump also requires functional sodium ion transport and an external source of potassium ions. Iodide that is taken up by the thyroid is bound in an organic form within a few minutes so efficiently that the effective free iodide concentration in the gland is very low. The clearance of iodide into the thyroid is greater than the combined effects of organic transformation of iodide and its efflux, therefore the iodide concentration in the thyroid is always greater than in the blood.

Following iodide concentration it is oxidised; the reaction has a high redox potential and the only agents that are capable of oxidising at physiological pH 7 are hydrogen peroxide and oxygen. The iodination of Tg is catalysed by thyroperoxidase (TPO) (Taurog *et al.*, 1970) and it is essential for iodothyronine hormone formation within the thyroglobulin molecule (DeGroot *et al.*, 1977, Nunez *et al.*, 1982, Hosoya and Matsukawa 1975). Originally TPO was also described as the thyroid microsomal antigen but Czarnocka *et al.*, (1985) have shown that the thyroid microsomal antigen and TPO are identical. The sequence is comprised of ~2799 nucleotides which code for a 933 amino acid protein (Kimura *et al.*, 1987, Libert *et al.*, 1987, Seto *et al.*, 1987), in the human and the gene is located on the short arm of chromosome 2 (de Vijlder *et al.*, 1988). There is a hydrophobic sequence towards the C-terminal portion of the molecule which traverses the plasma membrane and anchors the molecule to the apical surface of the thyroid cell. There is a

shorter form of TPO cDNA arising by an alternative splicing of the primary transcript resulting in a 171bp deletion giving a TPO molecule which is 57 residues shorter, the levels of which vary in individuals (Kimura *et al.*, 1987 & Barnett *et al.*, 1990). The molecular mass under reducing conditions for the full polypeptide is 103-110 kDa (Banga *et al.*, 1984 & 1985) but under non-reducing conditions the molecular mass is above 117 kDa (Hamada *et al.*, 1985) suggesting the existence of high molecular weight complexes in the native state. Porcine TPO has been shown to contain approximately 10% carbohydrate and there are five glycosylation sites on TPO, four of which are glycosylated (Rawitch *et al.*, 1990). The presence of hydrogen peroxide has been demonstrated on the follicular apical cell surface (Bjorkman *et al.*, 1981, 1984), although there is now evidence that TPO as well as occurring on the apical membrane, is also present in smaller amounts on the endoplasmic reticulum (Nilsson *et al.*, 1987, Alquier *et al.*, 1989).

There is some evidence that iodination of tyrosine residues may occur before the Tg molecule reaches the apical cell surface; this implies that oxidised iodine may chemically iodinate the Tg resulting in the condensation of iodinated tyrosine residues to form the hormones within the Tg molecule (Ofverholm *et al.*, 1984). The concentrated iodide ion is oxidised to the active species and is then incorporated into the thyroglobulin molecule to form monoiodotyrosine (MIT) and diiodotyrosine (DIT). It is these iodotyrosines that are condensed by an ether linkage to form T4 (thyroxine) or T3 (tri-iodothyronine) these structures are shown in figure 1.3 on the next page.

A 20 kDa N-terminal peptide is cleaved from thyroglobulin during the iodination reaction, but this remains attached via disulphide bonds (Dunn *et al.*, 1983). The iodoaminoacid composition of Tg depends on the levels of iodination of Tg (Rolland *et al.*, 1972); of the 120 tyrosine residues in Tg, only 25-30 are available for iodination to form iodotyrosines and of those only eight can couple to form the thyroid hormones. In Tg the number of thyroxine (T4) and tri-iodothyronine residues (T3) per mole of Tg increases linearly with enhanced iodine content. For example, in porcine Tg with an iodine content of 1.6%, the total number of iodothyronine residues is eight. This suggests that Tg has well defined sites for hormone synthesis and conversely the hormonogenic residues are located in an environment appropriate for iodination. In the human, normal iodination levels are represented by an upper value of 2-3 iodotyrosine residues per 660 kDa protein. This may appear wasteful, but Tg is the only known protein in the entire animal kingdom that is capable of forming the thyroid hormones, although, most proteins including thyroglobulin itself are capable of forming iodotyrosines when forcefully iodinated *in vitro*.

Exocytosis and colloid storage.

The newly synthesised Tg molecule is a 660 kDa dimeric glycoprotein composed of two identical, 330 kDa subunits with a sedimentation coefficient of 19S. Non-iodinated newly formed Tg is fully dissociable into 12S subunits by dissociating agents but the more

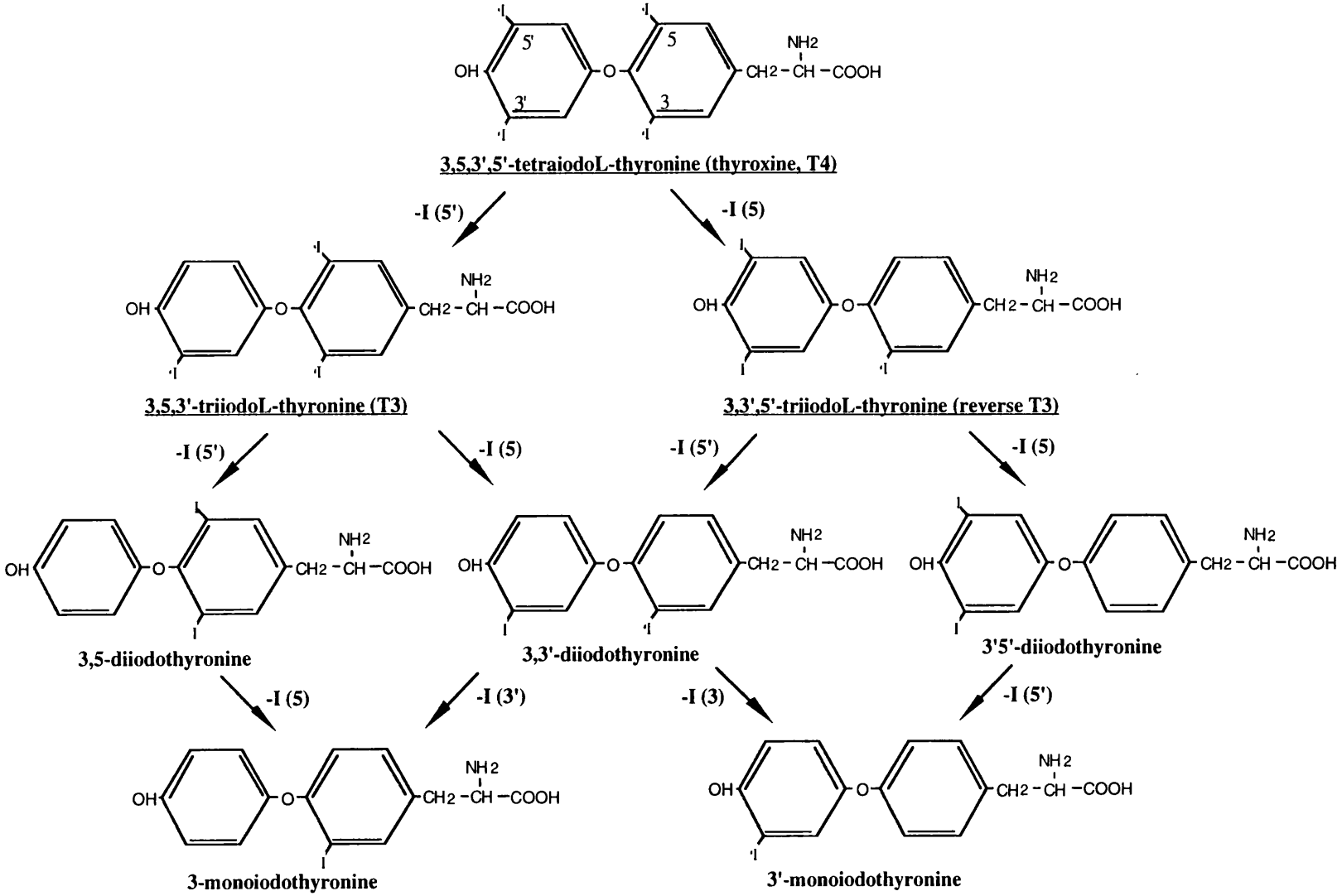


Figure 1.3. Structure of the iodothyronines.

iodinated the molecule becomes the more resistant to dissociation it becomes. Under prolonged thyroid stimulating hormone (TSH) administration there is a concomitant decrease in luminal size and Tg content of the gland. Conversely, elimination of TSH causes enlargement in luminal size and Tg content (Tarutani & Ui., 1968). Thyroglobulin passes through the Golgi complex to the apical pole of the thyroid cell. Here the final stages of migration into the colloid take place in exocytotic vesicles (Bjorkman *et al.,* 1974, Ekholm *et al.,* 1975a, Bjorkman *et al.,* 1976) whose contents are discharged into the lumen following fusion with the apical cell membrane (Ericson *et al.,* 1978). One major type of exocytotic vesicle identified in the apical region which has been shown to transport Tg (Bjorkman *et al.,* 1974) is typically of 150nm diameter with a smooth membrane. These vesicles have been shown to contain 18-19S Tg and have peroxidase activity (Bjorkman *et al.,* 1976) and may be found anywhere between the Golgi and the apical pole in normal thyroid glands. Thyroglobulin is then safely stored in the lumen until such times that hormone is required.

Virtually all the protein stored (>90%) in the colloid is 19S Tg the remainder is mainly 12S subunits associated into tetramers of 1300 kDa and hexamers of 2000 kDa whose function is unknown, (Smeds *et al.,* 1972); these authors have also shown that the thyroglobulin concentration of the thyroid has been measured between 100-400 μg/ml in the rat. It is the storage of such large quantities of Tg that allow the thyroid to maintain constant levels of thyroid hormone in the individual; this level of storage is achieved by the compaction of Tg molecules in the lumen. The diffusion of Tg from the follicle lumen has been shown to be very low (Loewenstein & Wollman, 1967) and this diffusion is controlled in part by TSH (Gerber *et al.,* 1985) but also by the viscosity of the Tg contained within the colloid. Tg in the colloid of bovine thyroid glands exists in two different forms of aggregation; soluble Tg which occurs as Tg subunits, monomeric, trimeric molecules or as small aggregates, or solid Tg (Herzog *et al.,* 1992). This is an almost insoluble form which exists as large globules which are resistant to common protein dissociation procedures such as EDTA and SDS but are susceptible to dissociation by trypsin. Most (97%) of the Tg in globules is covalently cross-linked via non-sulphide bonds but some is due to the formation of disulphide bridges. The iodine content of thyroglobulin in vesicles is higher (55 iodine atoms/ 12S subunit) compared to the soluble Tg (12 iodine atoms). It is this highly iodinated 19S Tg that is not completely dissociable into 12S subunits and the inter-molecular cross-linking may be a result of dityrosine bridges which are formed during the iodination of Tg. The storage of Tg in such forms allows the storage of high concentrations of thyroglobulin in an osmotically inert form. It has been suggested that newly exported and therefore uncomplexed Tg is internalised and hydrolysed first and that extracellular compaction might be the structural basis for sorting freshly exported Tg which is able to undergo rapid endocytosis, whereas previously exported Tg is utilised only when large quantities of hormone are required (Schneider, 1964). Observations with thyrocytes have shown that Tg is taken up primarily by a micropinocytotic process utilising coated vesicles (Bernier-Valentin *et al.,* 1991, Wollman 1989) and may be mediated by a low affinity

binding site for Tg (Lemansky & Herzog 1992). However this requires the availability of single Tg molecules so that mechanisms may exist to dissociate globular Tg to facilitate micropinocytosis. A schematic representation illustrating the orientation of the thyroid cell and Tg production is shown in figure 1.4 below.

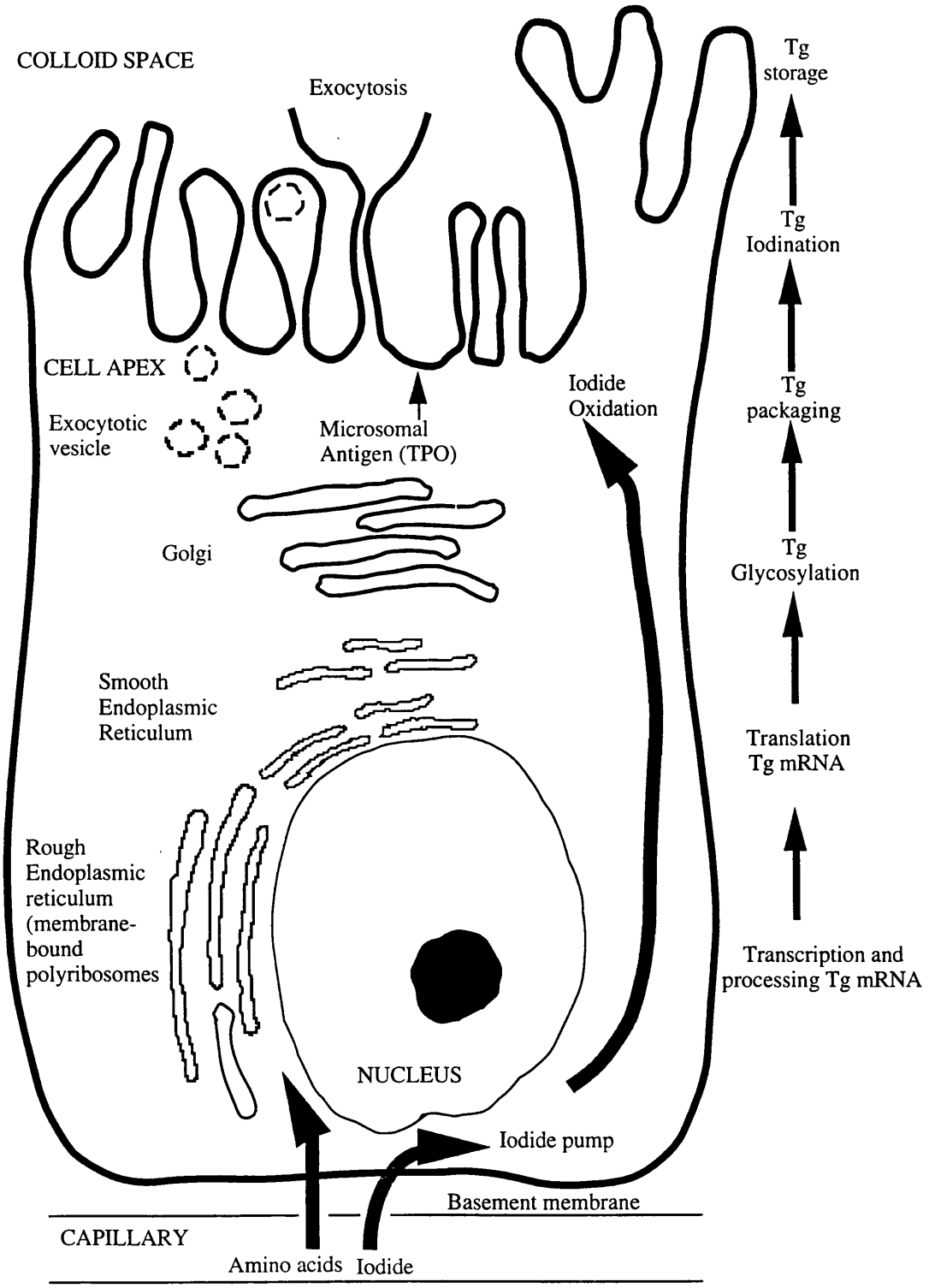


Figure 1.4. Schematic representation of a thyroid cell illustrating the synthesis and transport of thyroglobulin.

A) Hormone formation.

Endocytosis.

Transcytosis is the vesicular transfer of molecules in the cytoplasm of polarized cells from one plasma membrane to another and this has been shown to be the prerequisite for the appearance of whole thyroglobulin in the circulatory system (Herzog *et al.*, 1983, 1984), whereas endocytosis and the formation of lysosomes results in the degradation of Tg to form the hormones. When required, Tg is engulfed at the apical region to form endocytic vesicles or colloid droplets which then are able to fuse with lysosomes (Seljelid & Nakken, 1968). There are two endocytic pathways utilised by the follicle cell for the recovery of Tg, these are macro and micropinocytosis; micropinocytosis appears to be the major pathway in the normal thyroid (Sejelid *et al.*, 1970) and the chronically stimulated gland (Rocmans *et al.*, 1978). Macropinocytosis is characterised by the development of pseudopods at the apical cell surface and only seems to occur under strong TSH stimulation.

Micropinocytosis is rapidly detected following TSH stimulation and is characterised by the follicular cells developing large pseudopods; again this is located to the apical margin and their size, number and appearance rate are directly related to the levels of TSH stimulation (Engstrom *et al.*, 1981). The colloid droplets may be up to 3μm in diameter and are surrounded by a single unit membrane (Sejelid. 1967), endocytosis of these colloid droplets is dramatically increased following TSH stimulation.

The effect of TSH stimulation on endocytosis may be transitory. Romagnoli & Herzog (1991) have shown that the effect of TSH on endocytosis only lasts up to 16 min, whereas the effect of TSH on transcytosis is much longer lasting (>1h). Analysis of Tg before and after transcytosis shows that the molecular weight is not altered, suggesting the vesicles are able to bypass the lysosomal compartment. However there are differences in the molecular weight of Tg in the follicular lumen relative to that in the circulation (Schneider *et al.*, 1985) probably occurring as result of partial hydrolysis on reaching the circulation.

Vesicles are formed by invagination of the plasma membrane over its entire luminal surface. The vesicles formed appear homogeneous in size and morphology, and lack peroxidase (Tice *et al.*, 1974, Bjorkman *et al.*, 1976). Drugs that inhibit microtubule formation also affect macropinocytosis and thyroid hormone secretion induced by TSH (Wolff & Bhattacharyya., 1975, Neve *et al.*, 1970), but not micropinocytosis. These drugs are also able to influence the polarity of the follicular cell by affecting the position of the Golgi and the exocytotic vesicles. Given the heterogeneity of Tg in the colloid and the homogeneity of Tg in the colloid vesicles, is there a selectivity in endocytic pathways? Macropinocytosis appears not to be selective as red blood cells (RBC) in hyperblastic thyroids can be engulfed by pseudopods following TSH stimulation. However it has been shown that the degree of iodination and the level of aggregation of the protein could affect

endocytosis suggesting that micropinocytosis may occur via membrane receptors. Thus selective micropinocytosis may occur at preferential sites along the apical membrane, possibly where the binding sites for Tg are, and therefore the Tg in vesicles may have been selected on a qualitative basis.

Thyroglobulin proteolysis and hormone secretion.

The thyroid follicular cell contains lysosyme vesicles of 0.1-1.0 μm in diameter containing phosphatase (Wetzel *et al.*, 1965). They also contain hydrolytic enzymes which are active at acid pH, which include: glycoside hydrolases (Chabaud *et al.*, 1971), cathepsin D (Dunn *et al.*, 1982a), thiol endopeptidases (Dunn *et al.*, 1982b), exopeptidases (Dunn *et al.*, 1971) and thiol proteases which specifically release T4 from Tg (Nakagawa *et al.*, 1985). Colloid droplets have been shown to fuse with lysosomes 5-10 minutes after formation (Seljelid *et al.*, 1967, Engstrom *et al.*, 1981) to form phagolysosomes. The efficient release of thyroid hormone requires that the pH in the phagolysosomes is acidic. The membrane is impermeable to ionic species and also contains an ATP proton pump which probably helps to maintain the acidic pH (Fouchier *et al.*, 1984). In culture, thyroglobulin degradation is maximal although not complete at pH 3.7-4.0 (Dopheide *et al.*, 1969) and negligible at pH7 (Lamas & Ingbar., 1978) although low levels of iodination result in increased susceptibility to protease attack. Using purified porcine lysosomes as a source of porcine proteases and thiol reagents results in greater than 85% release of iodoaminoacids from either human or rat Tg (Yoshinari & Taurog., 1985). Enzymatic release of iodoaminoacids from Tg yields equimolar amounts of T3 and T4 which is different from the molar ratio of T3 and T4 found in the Tg molecule (Vandenbroucke *et al.*, 1972). The iodinated compounds that leave the thyroid gland as well as the T3 and T4 hormones include whole Tg, inactive reverse T3, monoiodotyrosine (MIT) and diiodotyrosine (DIT).

De-iodination within the follicle cell.

Following Tg hydrolysis by lysosomal proteases, the iodotyrosines MIT and DIT are almost totally deiodinated by microsomal iodotyrosine dehalogenase (Lissitzky *et al.*, 1982), which contains a flavoprotein shown to be activated *in vitro* by NADP-H ferrodoxin reductase (Goswami & Rosenberg., 1979, 1981). This system has no effect on the iodothyronines. As a result , although MIT and DIT leave the thyroid they are only present in the blood in trace amounts (Shalom *et al.*, 1966), though this can be increased by the thyroid hyperplasia associated with Graves' disease (Nelson *et al.*, 1974). Iodide liberated in the above reactions is largely re-utilised *in situ* for fresh hormone synthesis and provides 2-3 times the amount of iodide that is scavenged from the bloodstream (DeGroot & Bohlar., 1971). Some released iodide is secreted in amounts comparable to the hormonal iodide, that is 40-50μg per day in a normal healthy individual out of a daily dietary intake of about 200μg. Iodide leakage varies and is related to iodide intake, in that leakage prevents excessive hormone formation. This process has been shown to be partially controlled by TSH (Fayat & Hovsepian., 1977). In addition to T3 and T4, small amounts of T2 (di-iodothyronine) are generated from Tg

hydrolysis and T3 de-iodination released from the thyroid (Laurberg, 1984). Tri-iodothyronine is secreted more rapidly than T4, probably due to the faster liberation of T3 from Tg in proteolysis since T3 has been shown to be located in the C-terminal zone which is very sensitive to hydrolytic cleavage (Marriq *et al.*, 1983). Thus the influences on the pattern of T3 and T4 secretion, include the iodothyronine content of Tg, rate of colloid endocytosis, the levels of intra-thyroidal de-iodination of MIT and DIT and the differential rates of liberation on hydrolysis from Tg.

As mentioned previously, Tg is also secreted from the thyroid, giving rise to concentrations of between 5-32ng/ml in the blood in normal human and 100 ng/ml in the rat (VanHerle *et al.*, 1979). However these levels can be substantially elevated in disease and as a result of TSH stimulation. Thus in the rat, 7% of the Tg produced in a day can be released into the circulation and hydrolysis of this Tg in the periphery may be an additional source of hormones as well as a source of normally sequestered and therefore potentially pathogenic epitopes (Izumi & Larsen., 1978). In most thyroid diseases, serum levels of Tg are raised due to leakage from the gland; this may be due to the pathology resulting in alterations to the tight junction status of the gland as is believed to occur in Graves' disease or destruction of the follicular structure as occurs in thyroiditis (VanUijen & VanDijk., 1983). The presence of TSAb in Graves' disease also leads to an increase in the output of undegraded Tg. It has been demonstrated by Schneider *et al.*, (1983) that the iodine content of normal serum Tg is low and that this has a composition different from that seen in the gland perhaps because poorly iodinated Tg is not susceptible to proteolysis. In both diseased (thyroid cancer) and normal glands the degree of iodination of Tg in the gland is always much higher than in the serum and since there is no significant destruction of the gland in thyroid cancer; it is suggested that the high levels of poorly iodinated Tg in thyroid cancer comes from the tumour. The mean half life of thyroglobulin in the periphery is approximately 4.4 h (Feldt-Rasmussen *et al.*, 1978).

1.2 Regulation of thyroid function.

Thyroid stimulating hormone (TSH).

The main regulation of the thyroid gland is via thyrotrophin or thyroid stimulating hormone (TSH). TSH synthesis and secretion are subject to stimulation by hypothalamic thyroid releasing hormone (TRH) and to negative feedback by T3 (Larsen., 1982). The action of TSH is mediated by binding to its receptor and signalling via the cyclic AMP (cAMP) cascade. The main thyroid specific control of TSH thyroid regulation is exerted by iodide itself. Organic iodine is able to inhibit the TSH induced proliferation of rat FRTL-5 thyroid cells in a dose-dependent manner and the effect is believed to be mediated by both the cAMP-dependent and independent pathways (Tramontano *et al.*, 1989, Becks *et al.*, 1988). As well as thyroglobulin and TPO, the TSH receptor has been shown to be a main focus

in autoimmune thyroid disease. Its function may be activated by stimulating antibodies as occurs in Graves' disease or inhibited by the binding of anti-TSHR blocking antibodies as in atrophic myxoedema.

The human TSH receptor is a glycoprotein of 744 amino acids belonging to a family of G-protein coupled receptors which include the luteinising hormone - choriogonadotrophin receptor. They typically contain a large extracellular domain (398aa in the TSHR) which have multiple sites for N-glycosylation (6 in the human TSHR). The remaining amino acids form seven membrane spanning domains which have sequence homology with other G-protein coupled receptors. The extracellular domain exhibits a 25 residue leucine rich repeat sequence (Parmentier *et al.*, 1989, McFarland *et al.*, 1989) which has the potential to form amphipathic helices common in other transmembrane proteins; these are believed to form domains which have been implicated in protein/protein interactions. (Roth., 1991). The receptor is believed to exist at the cell surface as a heterodimer as result of post-translational cleavage (Vassart & Dumont., 1992). The transmembrane segments between LH/CG receptor and TSH receptor show a 70% similarity, whereas the extracellular domains only display a 40% similarity, suggesting that the specificity for hormone binding is encoded in the amino terminal extracellular portion while the transmembrane domain probably plays a role in intracellular signalling although the precise nature of this has not yet been elucidated.

As already mentioned, the TSHR constitutes the target for autoantibodies in certain thyroid diseases. Studies comparing the effects of TSH and thyroid stimulating antibodies (TSAb's) on cells transfected with the TSH receptor modified by site directed mutagenesis conclude that the amino-terminal segment of the TSHR, residues 1-89, is probably involved in the binding of antibodies from Graves' patients (Wadsworth *et al.*, 1990, Tahara *et al.*, 1991). A sequential search for the autoepitopes on the TSHR have proven negative (Libert *et al.*, 1991), suggesting that the epitopes that serve as targets for autoantibodies are non-sequential *i.e.* are discontinuous. Conflicting results have arisen over the binding regions of antibodies to the cytoplasmic region of the TSHR. Rabbit antibodies to segments 172-202 and 341-370 display potent TSAb activity and do not interfere with TSH binding to the receptor (Endo *et al.*, 1991). Nonetheless residues 172-202 have been shown to contribute to the TSH binding domain (Nagayama *et al.*, 1991) and residues 341-370 are within a segment of the TSHR that can be deleted without altering the ability of TSAb to stimulate the TSHR (Wadsworth *et al.*, 1990); it cannot be excluded that experimentally generated antibodies may be against or may activate by binding to epitopes other than those recognised by TSAb's. Two other studies have shown that segments 333-343 and 352-366 are recognised by thyroid stimulating antibodies (Mori *et al.*, 1991, Takai *et al.*, 1991). However the segments fall within a region whose deletion is fully compatible with TSHR stimulation by TSAb's (Wadsworth *et al.*, 1990); these may therefore represent good examples of epitopes generated experimentally that may not be involved in pathogenicity.

TSH is able to activate one of two regulatory cascades. In the human thyroid, TSH activates the cAMP and Ca^{2+} PIP_2 cascade, although the latter requires concentration of TSH, 10x that required by the cAMP cascade (Laurent *et al.*, 1987). By comparison, the cAMP cascade in the dog thyroid and the thyroid derived cell line, FRTL5, is activated by TSH only at physiological concentrations (Dumont *et al.*, 1971). For the activation of either of these cascades the receptor must be bound and therefore activated by its ligand. The activation step involves the exchange of GDP by GTP and the dissociation of the G protein into its subunits α and $\beta\gamma$; the α subunit then binds to GTP stimulating the activation of the effector enzymes adenylate cyclase which generates cAMP from ATP and PIP_2-phospholipase C which generates 1,4,5-PIP_3 and diacylglycerol from phosphatidyl inositol 1,4,5-triphosphate. Thyroid stimulating antibodies at the maximal concentrations known to exist in Graves' disease are only able to stimulate the cAMP pathway. In the human thyroid, cAMP mediates the effects of TSH on iodide trapping and the secretion of thyroid hormone via the phosphorylation of serine and threonine on as yet unknown proteins. Whilst Ca^{2+} and diacylglycerol stimulate iodination and synthesis of the thyroid hormones, Ca^{2+} effects are believed to be mediated by calmodulin via calcium/calmodulin dependent kinases.

TSH stimulation of thyroid iodide transport is delayed as it requires production of new mRNA and therefore protein synthesis. It has been demonstrated by Nagayama *et al.*, (1989) that TSH induces TPO mRNA transcripts and Tg mRNA transcripts in cultured human thyroid epithelial cells in a concentration- and time-dependent manner. The same results were achieved by the addition of 8-bromo-cAMP a cAMP analogue, these effects are blockable by cycloheximide, indicating that the levels of mRNA transcripts in these cells was due to newly synthesised protein. This effect can be repeated by the addition of forskolin which increases levels of cAMP (Collison *et al.*, 1989). Interestingly the addition of IFN-γ to human thyroid epithelial cells, whilst under TSH stimulation, inhibits the increase of thyroid peroxidase (TPO) content of the cell whilst enhancing the expression of DR (this will be discussed later).

In the dog and pig, activation of the calcium/PIP_2 cascade increases the efflux of iodide. This occurs at the apical membrane and results in the increased transport of iodide from the cell to the lumen thus increasing the supply of iodide at the apical membrane available for iodination (Nillson *et al.*, 1990). Protein iodination is limited by the physiological supply of iodide, but it can be increased by greater production of H_2O_2 at low iodide concentrations in human thyroid cells and at high concentrations in dog thyroid cells. As previously mentioned the thyroid hormones in thyroglobulin are derived from the oxidative coupling of iodotyrosine residues and these in turn depend on the iodide supply and the generation of H_2O_2. Thyroperoxidase is the enzyme that is involved in the iodination and oxidative coupling of Tg and expression of this gene is controlled by TSH through cAMP (Gerard *et al.*, 1989 & Nagayama *et al.*, 1989). Therefore regulation of iodination and hormone

synthesis depends on the generation of H_2O_2 which in the thyroid is controlled via the peroxidase regulated by cAMP. Thus TSH induces both an accompanying increase in peroxidase activity (Magnusson & Rappaport., 1985) and TPO mRNA levels (Damante *et al.*, 1989), an effect can be mimicked by cAMP analogues.

TSH as well as insulin and insulin like growth factor 1 (IGF-1) regulate thyroglobulin gene expression at the transcriptional level (Lee *et al.*, 1991, Van Heuverswijn *et al.*, 1984, Santiseban *et al.*, 1987) and translational level (Davies *et al.*, 1978). In thyroid cells that have been deprived of TSH, addition of TSH agonists or cAMP agonists cause a rapid increase in transcription after one hour (Gerard *et al.*, 1989). Thus in the fully differentiated thyroid cells rapid stimulation of thyroglobulin transcription is probably achieved by the phosphorylation of existing proteins in the cell.

Within the Tg gene there is a region of 250 base pairs upstream from the transcription start site which is implicated in the stimulation of Tg gene transcription by cAMP. Thyroid transcription factor-1 (TTF1) is known to interact with three sites in this gene segment and this may act as a direct target for cAMP regulatory effects (Guazzi *et al.*, 1990). In the rat (Dere & Rappaport 1986), dog (Van Sande *et al.*, 1989) and human thyroid cells (Roger *et al.*, 1988) in primary culture, TSH is also able to stimulate proliferation of these cells; this effect again may be mimicked by cAMP analogues or other agents that enhance cAMP accumulation in the cell such as forskolin.

1.3 Autoimmune thyroid disease.

Autoimmune thyroid diseases are characterised by sensitisation to the thyroid autoantigens *i.e.* thyroglobulin, thyroid peroxidase and the thyrotropin receptor. Other antigens have been described but are believed not to play a role in the induction and maintenance of autoimmune thyroid disease. Between the different autoimmune conditions associated with the thyroid the clinical features are varied. In Graves' disease there is stimulation of the gland by anti-TSHR antibodies which leads to hyperthyroidism. In Hashimoto's thyroiditis the inflammation of the gland leads to destruction and goitre. The coexistence of anti-TSHR antibodies can cause the receptor blockade producing the atrophic variant of thyroiditis, primary myxoedema and hypothyroidism.

1.3.1 Graves' disease.

Graves' disease is a familial autoimmune disease, more correctly classified as a syndrome that has variable expression. Its symptoms include goitre, hyperthyroidism and ophthalmopathy and the presence of one or more of the conditions determines a positive diagnosis. The most common feature is hyperthyroidism (over-production of thyroid

hormone) usually associated with goitre, in which the thyroid displays diffuse hyperplasia, which is a thickening of the normal single cell follicular wall. The disease, in common with autoimmune diseases in general, is more prevalent in females than males where the ratio is approximately 4 to 5:1 (females : males) (Furszyfer *et al.*, 1972, Barker & Phillips 1984). Hyperthyroidism is not an uncommon condition, the greatest incidence being in the geriatric population .

There are documented cases of families affected by what we now know to be Graves' disease from over 100 years ago and many subsequent studies have demonstrated this familial incidence of Graves' disease. The first reported study was the Bartels study in 1941 but many other studies since then have upheld this view (Bartels *et al.*, 1941, Heimann *et al.*, 1966). Since the recognition of Graves' as an autoimmune disorder, familial studies have concentrated on the incidence of autoantibodies. It has been shown that antibodies to thyroglobulin, TPO and the gastric parietal cell occur with increased frequency in families where one member has been diagnosed with Graves' disease. There is also an increased co-incidence of other autoimmune diseases such as type I diabetes and Addison's disease which occur with a greater incidence than that observed in the general population. There is also an increase in non-endocrine autoimmunity such as rheumatoid arthritis, myasthenia gravis, Sjögrens syndrome and vitiligo.

HLA associations have been reported for Graves' disease although it should be noted that no HLA antigens have 100% associations with Graves' disease or any other autoimmune disease. Thus there are obviously other factors which play as important a role in the pathogenesis of autoimmune disease, which include environmental factors such as pathogens, diet and stress. In Caucasian populations, DR3 is the most frequently reported HLA antigen in association with Graves' disease (McGregor *et al.*, 1980); in Japanese populations HLA-Bw35 is associated with Graves' (Sasazuki *et al.*, 1977) whereas in Chinese populations the associated antigen is HLA-Bw54. Class II is a HLA gene product gene product which in under normal conditions is only expressed on cells of lymphoid lineage such as B-cells, dendritic cells and other so called 'professional' antigen presenting cells. Class II serves the function of presenting exogenous antigen to class II restricted T-cells and thus initiating an immune response. In recent years the traditional view on the restricted expression of class II has been challenged. Class II expression has now been demonstrated on many non-lymphoid cells, such as thyroid cells, (Bottazzo *et al.*, 1983) taken from the thyroids of patients suffering from autoimmune thyroid disease; it is also inducible by the presence of cytokines such as IFN-γ. It has been postulated that the presence of class II molecules on the surface of thyroid cells may permit the presentation of TSHR as an antigen and thus lead to the production of autoantibodies. It is not known if class II expression causes autoimmunity or is a consequence of established disease. This will be discussed in more detail later in the text as a possible genetic defect of the thyroid gland and its possible implications. Other factors that have been implicated in pathogenesis

include stress and the effect that it may have on the immune system. There is evidence that stress can lead to increased susceptibility to infections and cancer in the human. In the laboratory rat the same effect is seen when they are stressed by conditions of isolation or overcrowding. One of the major factors believed to be important in the pathogenesis of Graves' disease and Hashimoto's thyroiditis is an excess of iodide in the diet.

Iodine is required for normal thyroid function and the formation of the thyroid hormones. In the United States the recommended daily allowance is 0.15mg, but it has been shown that many Americans ingest more than 1mg/day these high levels coming from milk and its products, cereals, meat, fish, poultry and iodized salt. The maximum toxic dose is estimated to be up to 2mg/day but this is often exceeded. Amiodarone is a widely used antiarrhythmic drug that is structurally similar to the thyroid hormones and has a high iodine content (37% of its molecular weight). Heart patients receiving the drug regularly receive 6-12mg/day of free iodide as a breakdown product of the drug, these patients show significant increases in serum T4 and rT3 (inactive T4 metabolite) (Melmed *et al.,* 1981, Franklyn *et al.,* 1985) levels and a concomitant decrease in serum T3 levels. Amiodarone may also have a direct binding effect on the pituitary by binding to T3 receptors and directly augmenting secretion of TSH (Franklyn *et al.,* 1985, 1987, Norman & Lavin 1989). The induction of thyroid disease is related to the iodide content of the drug and it has been shown that the rates of thyroid dysfunction and thyrotoxicosis in the user population are higher in areas of endemic iodide deficiency than they are in areas of normal iodide intake (Martino *et al.,* 1984); but there is no clear relationship between the development of disease and dose or duration of treatment. There is an absence of ophthalmopathy and thyroid stimulating antibodies in most cases of thyrotoxicosis induced by amiodarone which may argue against iodine induced activation of autoimmune Graves' disease. In areas where iodine intake is normal, hypothyroidism is more common than thyrotoxicosis, it typically occurs early in drug treatment and is more common in patients already possessing microsomal or thyroglobulin autoantibodies. There is some disagreement on what proportion of the clinical problems described are a direct result of amiodarone therapy in the study already mentioned; but in both high and low intake areas, the incidences of anti-microsomal antibodies were much higher than those of the normal population. However of those patients exhibiting amiodarone induced hypothyroidism, not all exhibited anti-microsomal or anti-Tg autoantibodies.

Hyperthyroidism is clinically diagnosed by the measurement of serum concentrations of TSH, T4 and T3; both TSH and T4 are elevated in hyperthyroidism and T3 is almost always enhanced. The vast majority of patients who have hyperthyroidism associated with Graves' disease present with complaints of nervousness, weight loss, sweating and palpitations and they may also describe an intolerance to heat and emotional lability. Patients may also present with an accompanying ophthalmopathy and exhibit stare. They may also have a finger tremble and goitre. Not every patient presents with a complete list of these features

and the combinations can vary; some patients may not even present with goitre. A feature which is highly characteristic of hyperthyroidism is the warm moist skin and heat intolerance, the symptoms being due to the dilatation of blood vessels in the skin. The patients pulse is typically strong and often rapid even during sleep, also due to the dilatation of the blood vessels. The common symptom of weight loss is often accompanied by an increased appetite, anorexia can occur in severe examples and abdominal examination may reveal splenomegaly. This is part of the general lymphoid hyperplasia and histological examination reveals the formation of germinal centres in the thyroid, indicating B-cell hyperplasia in response to pathogenesis.

Thyroid associated ophthalmopathy is a progressive eye condition that frequently occurs in Graves' disease and can occasionally occur in Hashimoto's thyroiditis. The disorder is usually two stage, the first occurring in a majority of patients with Graves' disease and a small number of Hashimoto's patients. Antibodies and $CD4^+$ T-cells are directed towards 64 kDa membrane antigens which are expressed in membrane fractions of eye and cardiac muscle (Salvi *et al.*, 1988, Salvi *et al.*, 1991, Zhang *et al.*, 1992a, Ross *et al.*, 1993, Zhang *et al.*, 1992b) and thyroid cells (Dong *et al.*, 1991). The second stage occurring in about 25% of Graves' patients and 2% of Hashimoto's patients, is probably due to cytotoxic antibodies and cells to a 35 kDa antigen. Thyroid associated ophthalmopathy may in the future be a potential candidate for monoclonal antibody therapy, possibly anti-CD4.

There are a number of treatments for hyperthyroidism. Iodide and blockade of β-adrenergic receptors are only used in mild cases as long term administration of iodide may lead to exacerbation of the disease. For more severe cases, partial surgical section of the thyroid can be done, but it is accepted that patients younger than 40 years old who have a good life span are likely to become hypothyroid in the future and the preference here is to use of thionamide anti-thyroid drugs. Two are commonly used today, propylthiouracil and methimazole. Both interfere with the synthesis of thyroid hormones but propylthiouracil reduces the amount of peripheral de-iodination of T4 and T3 which is enhanced in hyperthyroidism (Abuid & Larsen 1974). Administration of 300 mg propylthiouracil or 30 mg methimazole per day is sufficient to control a clinical hyperthyroid state within eight weeks of diagnosis. It is accepted that relapse after a course of therapy means that the potential of remission after a second course of therapy is low and therefore other alternative therapy is considered.

1.3.2 Autoimmune Thyroiditis.

Hypothyroidism results from decreased thyroid hormone production and reduced levels of thyroid hormone in the blood, its severity varying greatly from the sub-clinical condition where serum T3 and T4 levels are normal, to overt hypothyroidism with abnormal function of organ systems. In common with other autoimmune diseases it is more frequently found in women than men; it has been estimated that 8-10% of women and 1-2% of men have sub-clinical hypothyroidism. The existence of a possible autoantigen being involved in Hashimoto's thyroiditis was first demonstrated by Roitt *et al* ., (1956). Antibodies in the sera of patients were shown to bind a thyroidal protein, namely thyroglobulin; following this initial discovery the existence of other autoantibodies including those against TPO have been demonstrated (Bogner et al 1984). Those antibodies against the thyroid microsomal antigen are cytotoxic (Bogner *et al.,* 1984) whilst anti-Tg antibodies do not have any biological action. Thyroiditis patients have also been shown to have antibodies that are able to inhibit TSH binding to its receptor on thyroid cells, thyroid adenylate cyclase activity, stimulate thyroid growth and block cAMP stimulation of iodine metabolism (Valente *et al.,* 1983, Takasu *et al.,* 1984, 1987). In the normal subject, approximately 100 µg/day T4 is produced compared to 20 µg/day in the hypothyroid patient; the effective serum concentration is 8 µg/dl and 2 µg/dl respectively, a 75% decrease in the level of the hormone. T3 production is less affected, 30 µg/day being produced in the normal subject, compared to 10 µg/day in the hypothyroid patient. As the disease progresses, there is compensatory TSH secretion which can restore thyroid function. The commonest cause of thyroidal hypothyroidism is autoimmune thyroiditis although hypothyroidism may result from a pituitary or hypothalamic deficiency.

Autoimmune thyroiditis presents in an atrophic, non-goitrous form (primary myxoedema) and a goitrous form Hashimoto's thyroiditis. The primary myxoedema is characterised by the destruction or atrophy of the thyroid follicular architecture accompanied by lymphocytic infiltration, whilst the latter is characterised by thyroid follicular hyperplasia, lymphocytic infiltration, fibrosis and the formation of germinal centres. Both forms result from antibody and cell-mediated interactions (Woolner *et al.,* 1959). Patients can remain euthyroid or have sub-clinical or overt hypothyroidism whilst some exhibit defects in thyroid hormone biosynthesis for example in iodide organification a considerable sensitivity to inorganic iodide (Vagenakis & Braverman 1975). There are many instances where increased thyroid disease can be related to the presence of iodide. Braverman *et al.,* (1971) looked at the effect of the administration of iodide (180mg/day) to patients suffering from Hashimoto's thyroiditis as defined by the presence of autoantibodies with normal thyroid function (serum T4 and TSH normal). These patients became hypothyroid (drop in serum T4 and rise in TSH) on exposure to this level of iodide which was reversed 5 weeks after the cessation of treatment. In a similar study by Boukis *et al.,* (1983), goitrous patients were studied for the effect on thyroid function of iodized oil injections (0.5mg). Within 6 months, 43%

of patients had developed anti-microsomal antibodies and 23% anti-Tg antibodies. For most of the patients in the study serum T4 and TSH levels were within their normal ranges and goitre sizes decreased. A small number of patients developed hyperthyroidism as characterised by elevated serum T3 and T4 as well as anti-microsomal and anti-Tg autoantibodies. T-cells from thyroiditis patients are sensitised against thyroid antigens producing cytokines when exposed to thyroid antigens; the addition of normal T-cells inhibits cytokine production (Okita *et al.,* 1981); thus there is the possibility of an antigen-specific defect in suppressor T-cell function in these patients. In common with most autoimmune diseases autoimmune thyroiditis is more common in females than in males and there is a hereditary factor within families.

Two further studies suggest a fundamental difference between the normal and diseased gland in the response to iodide. Fragu *et al.,* (1985) looked at patients with severe autoimmune thyroiditis, whose diet was supplemented with 0.5mg/day iodide. In contrast to euthyroid patients, who showed an increased content of iodine in their thyroid glands but no effects on serum T4, T3 or TSH, the Hashimoto's patients showed both increases and decreases in the iodide content of their gland. Some patients had reduced serum T4 levels and elevation of TSH, while in others, serum T4 levels increased and TSH decreased. A study performed by Tajiri *et al.,* (1986) indicated that in patients with autoimmune thyroiditis, excess dietary iodide induced clinical hypothyroidism. These patients with clinical hypothyroidism had antibodies to microsomal antigen and/or Tg and their dietary intake of iodide was between 2-3mg/day. On reduction of dietary intake of iodine, half of the patients reverted to a euthyroid status and those who did not had too severe thyroiditis for there to be a benefit. Those who reverted then increased their dietary intake again, all showed elevated serum TSH and some developed symptoms of clinical hypothyroidism. This contrasts with the control group ingesting the same level of iodide who exhibited no evidence of thyroid dysfunction and only slightly elevated serum TSH levels. Thus iodine can be shown to aggrevate the thyroid function in those individuals who already have Hashimoto's thyroiditis at levels less than those well tolerated by normal individuals.

Hashimoto's thyroiditis has been shown to be associated with HLA-DR4 (Weissel *et al.,* 1980) & DR5 (Farid *et al.,* 1988) whereas the atrophic variant, primary myxoedema has been shown to be associated with the expression of HLA-DR3 (Farid *et al.,* 1988), in Chinese populations development of Hashimoto's thyroiditis has been shown to be associated with HLA-DRw9 (Hawkins *et al.,* 1987). These associations have recently been updated by using DNA probes for DR and DQ genes and it has been shown that DR4 and DR5 are in linkage disequilibrium with DQw7 which is encoded by the DQB1 gene, in Hashimoto's thyroiditis. The presence of DQw7 confers a relative risk of 4.7 and has been shown to be present in 56% of Hashimoto's patients compared to 21% of control patients observed in a particular study by Badenhoop *et al.,* (1990). The adjacent DQA1 genes also display a significant association, DQA0201/0301 heterozygotes were found in 37%

of patients compared to 15% of controls. A subsequent study by Shi *et al.*, (1992) has refined the association and confirmed that DQA and DQB alleles are involved in susceptibility to thyroid autoimmunity. They propose that susceptibility is mediated through DQA0201 in linkage disequilibrium with DR3 or DQA0301 which is in linkage disequilibrium with DR4 and which is also a constituent of DQw7. It is suggested that over a 15 year period of study, these DR3 and DR4 linkage disequilibriums may account for nearly 82% of patients seen with Hashimoto's thyroiditis. These DQ alleles may determine the ability of peptides to bind to the groove of the Class II structure and thus be important in presenting pathogenic peptides to T-cells. It has been shown that DQ molecules are expressed by human thyroid cells when exposed to IFN-γ, this may allow subsequent antigenic presentation by those thyroid cells, this will be covered later in the text.

Once subclinical hypothyroidism is present as characterised by the presence of goitre, anti-microsomal and anti-Tg autoantibodies , chronic autoimmune thyroiditis results due to the progressive failure of the thyroid gland with time. In those patients with sub-clinical hypothyroidism, 5-25% a year develop overt hypothyroidism (Hayashi *et al.*, 1985). However autoimmune thyroiditis can occur transiently, usually post-partum in up to 3-5% of women up to one year after parturition (Amino *et al*; 1982, Jansson *et al.*, 1984a). The condition usually develops within three to six months of parturition and is characterised by enlargement of the gland and the presence of antibodies to the microsomal antigen; these symptoms usually subside within a few weeks or months. The hypothyroidism is often subclinical and may reoccur after subsequent pregnancies. The degree of infiltration of the thyroid gland is less than that seen in chronic autoimmune thyroiditis and there is no germinal centre formation. Hypothyroidism has also been shown to occur following radioiodine therapy treating Graves' patients for hyperthyroidism and following partial thyroidectomy for hyperthyroid Graves' disease.

The clinical manifestations of hypothyroidism are highly variable in their severity and their occurrence between patients. The thyroid hormones are required for the normal function of most of the body's organ systems and therefore a deficiency in these hormones can manifest in one or many organ systems of the human body. Although hypothyroidism is often diagnosed on the basis of serum T4 and TSH levels, some symptoms such as slow movement, coarse skin, a reduction in sweating, cold intolerance and slow reflex relaxation are important in the final diagnosis. Many patients are diagnosed as hypothyroid without any of these symptoms except by serum T4 and TSH levels. One factor that makes hypothyroidism very difficult to detect is the slowness of progression from the onset of disease; for example after thyroidectomy or the withdrawal of hormone in the hypothyroid patient, no symptoms of hormone deficiency develop for three to four weeks (Krugman *et al.*, 1975) and marked symptoms can take up to three months to occur.

Patients suffering from chronic autoimmune thyroiditis may exhibit defects in acid secretion

and vitamin B12 absorption, these patients may also have anti-parietal cell antibodies and later develop pernicious anaemia. Thus the symptoms occurring in hypothyroidism are many and varied, but this simply reflects the multifaceted role that thyroid hormone plays in the maintenance of normal body systems. Fortunately the treatment of hypothyroidism is relatively simple and effective. The therapy of choice is oral administration of T4 (thyroxine) and therefore a direct replacement of hormone. This therapy in the hypothyroid patient is lifelong and therefore education is also needed to ensure that the treatment is maintained by the patient.

1.4 Experimental Thyroid Disease

It has been known that autoimmunity is a cause of thyroid disease for nearly forty years, but many questions still remain regarding the pathogenesis of the autoimmune condition in the human. At the present moment there is no experimental model of Graves' disease although there are a number of satisfactory models for Hashimoto's thyroiditis, which have, over the years, helped illuminate several facets of the human condition. The existence of the animal model has aided the characterisation of the autoantigens involved, the identification of the immunological characteristics and the pathogenesis of the autoimmune condition.

There are two types of models available for the study of autoimmune thyroiditis, these are spontaneous, in which the disease develops without any intervention and experimentally induced, in which disease is produced by immunisation with thyroid antigens in adjuvant.

1.4.1 Spontaneous models of thyroiditis.

A) The obese strain chicken.

The obese strain (OS) chicken is the animal model which most closely resembles Hashimoto's thyroiditis, even though the avian immune system is unusual in having a bursa of Fabricus and an absence of lymph nodes. The OS strain was derived from the Cornell C strain (CS) (Cole, 1966) and the OS strain is highly susceptible to development of autoimmune thyroiditis, both strains share the same MHC but the OS spontaneously develops thyroiditis in 95% of the females following sexual maturity. Phenotypically the females are smaller than normal and have long downy feathers and have large subcutaneous fat deposits. Spontaneous autoimmune thyroiditis (SAT) occurs in the OS strain within the first three weeks of hatching and is characterised by a large infiltration of the thyroid gland, hypothyroidism and circulating AAbs (autoantibodies) to Tg from approximately 4 weeks of age. From 6-8 weeks of age germinal centres can be detected which replace functional thyroid tissue and eventually result in gland fibrosis. C3 and IgG deposits which form immune complexes consisting of Tg and anti-Tg AAbs which have been transferred from the mother are present at the basal lamina of the hatchlings thyroid glands

(Katz *et al.*, 1981, 1986, Kofler *et al.*, 1983).

It has been shown that T-cells from OS chickens are able to transfer the disease (Livezey *et al.*, 1981) but B-cells cannot (Polley *et al.*, 1981). OS strain T-cells are also able to transfer disease to the Cornell strain chicken which would otherwise not develop the disease. The CS chicken has the same MHC haplotype as the OS chicken but lack other immunological defects seen in the OS chicken. However pathogenic cells from the OS chicken are not able to transfer the disease to normal strains with the same MHC that have normal thyroid function (Wick *et al.*, 1990). Although B-cells are unable to cause disease themselves, it has been demonstrated that anti-Tg AAbs are able to cause pathological changes that can precipitate the disease in OS chickens (Jaroszewski *et al.*, 1978, Neu *et al.*, 1985). B-cells are intrinsically associated with the pathogenesis of SAT as early bursectomy (Bx) delays the onset and decreases the severity of disease; thus the disease has been shown to be mediated by both B and T-cells.

In common with Hashimoto's thyroiditis, the OS model develops a polyglandular organ-specific autoimmunity. Accompanying the reactivity to thyroid antigens there is also reactivity to other autoantigens from the gastric mucosa/adrenal gland and pancreas (Khoury *et al.*, 1982). The incidence of disease in the OS chicken appears to be linked to a generalised T-cell hyperactivity as indicated by an increase in T-cell proliferative responses, IL-2 production and CD25 expression (Schauenstein *et al.*, 1985), this is also accompanied by a generalised B-cell hyper-responsiveness which accords with the observation that bursectomy in the embryo before hatching reduces the resulting disease severity (Wick *et al.*, 1990). This effect can be replicated by the administration of cyclophosphamide 7-10 days after hatching and results in severe lymphocytic depletion and loss of antibody production. These effects are reversed following repopulation of the bursae with bursa cells (Rose *et al.*, 1981, Bacon & Rose., 1979). In contrast neonatal thymectomy (Tx) has the opposite effect in that it induces maximal thyroid infiltration and high titres of circulating autoantibody (Wick *et al.*, 1974). If this therapy is combined with the administration of turkey anti-chicken T-cell serum, which depleted the circulating T-cells, no SAT and no anti-Tg AAbs developed. Therefore this is evidence that T-cells might promote the appearance of disease or regulate immune cell function and therefore development of disease (De Carvalho *et al.*, 1982).

The disregulation of the OS chicken immune system is suggested by the observation that OS chickens show an imbalance between Th activity and T-cell regulatory activity (Wick *et al.*, 1985) also OS chickens have a deficiency in the thymic nurse cell population (Boyd *et al.*, 1984) which are sites of T-cell differentiation (Wekerle & Ketelsen 1980, Kyewski & Kaplan 1982), these cells express class II antigens and are involved in the processes of thymic selection. In the OS chicken, there is also a decrease in glucocorticoid production (Fassler *et al.*, 1986) and a deficiency in the neuroendocrine feedback control of the immune

systems response to cytokine. It is also possible that the presence of an endogenous virus ev22 in OS chicken thyroid glands (Wick *et al.*, 1985, 1986) may result in molecular mimicry of thyroid autoantigens which may cause inappropriate antigen specific T-cell activation and thus autoimmunity. These abnormalities together with disordered thymic maturation serve to ensure the incidence of autoimmune thyroiditis in the OS strain chicken is nearly 100% (Rose *et al.*, 1976).

In common with Hashimoto's patients, thyroglobulin that has been purified from CS chicken thyroid glands and when analysed for iodine content, shows a reduction of iodine content from 46 atoms/molecule (in the normal chicken) to 27 atoms/molecule even when those CS chickens have been provided with a normal level of iodine intake in the diet (Sundick *et al.*, 1991). This effect is also observed when the chickens are fed on a T4-supplemented diet which would be expected to reduce serum TSH and therefore reduce thyroid hormone secretion. This apparent abnormality of the thyroid gland appears to be present before thyroid infiltration begins. OS chickens injected at hatching with thyroglobulin have no mononuclear cell infiltration and no detectable autoantibodies at three weeks of age (Sanker *et al.*, 1985) but still have significantly reduced iodine/thyroglobulin ratios when compared to normal hatchlings. The iodine content of CS chicken thyroglobulin has been shown to be intermediate between that of normal chickens and that of OS chickens when maintained on a normal diet. The mechanism by which this defect in iodine metabolism modulates or causes thyroiditis is not known although it has been postulated that iodine levels may affect the immunogenicity of thyroglobulin. Highly iodinated thyroglobulin when injected into normal chickens induces a greater autoantibody response that by poorly iodinated thyroglobulin (Sundick *et al.*, 1987). This has also been demonstrated in the induced mouse model and this will be discussed later in the text. However it has been shown that the autoantibodies produced in the OS chicken react equally well with highly and poorly iodinated thyroglobulin (Sundick *et al.*, 1987); therefore the levels of autoantibody produced in relation to iodine content may be as a result of an effect on Th-cell function.

Although the OS chicken is a good model of human thyroiditis there are some differences; for example in the human, large amounts of inorganic iodide are demonstrable in the gland (Kivikangas *et al.*, 1970 & Jonckheer *et al.*, 1981) and this is not seen in the OS chicken. However this may reflect the fact that studies in man cannot be made before onset of disease so that it is difficult to ascertain the conditions in the gland prior to this; consequently iodination defects have only been described in clinical disease. It has been postulated that an abnormal iodine metabolism may promote thyroiditis through the formation of excess iodine radicals. Free radicals may cause thyroid cell damage directly or through the formation of reactive oxygen intermediates; damage may then lead to thyroid infiltration and the sensitisation of T and B cells. The antioxidant butylated hydroxyanisole has been shown to decrease the severity of thyroiditis in OS chickens (Bagchi *et al.*, 1990); this is evidence that damage by free radicals may be contributory to thyroid damage and may

indicate another role for iodine in the pathogenesis of autoimmune thyroiditis.

In the obese strain chicken, the level of circulating Tg is greatly increased in comparison to the level seen in the parental CS strain. It is interesting to note that neonatal thyroidectomy of OS strain chicken prevents the development of autoimmunity unless normal soluble chicken Tg is administered subsequently, when Tg AAbs can be detected, if the gland is then removed it abrogates established autoimmunity (Bigazzi & Rose 1975); thus there appear to be intrinsic thyroid gland abnormalities which mediate disease. Following on from this it has been shown that the threshold for MHC II induction and expression by interferon-γ in OS chickens is much lower than in normal strains and aberrant expression of class II on the chicken thyroid has been demonstrated (Wick *et al.*, 1984). These workers have shown that class II antigens appear in the immediate vicinity of class II positive infiltrating T-cells but they were unable to detect class II antigens on thyroid epithelial cells in OS chickens before infiltration begins. This may have important implications in the induction and/or perpetuation of the disease.

A study by Bagchi *et al.*, (1985) in the Cornell strain chicken has shown that dietary iodine can be causative of the development of thyroiditis. Hatchlings of the CS strain were supplemented with potassium iodide in the drinking water; by 6 weeks, autoantibodies to Tg, T3 and T4 were detected in a majority of hatchlings and by 12 weeks all had developed varying degrees of lymphocytic infiltration of their thyroid glands; this effect was directly related to the level of supplementation in the diet. Supplementation in normal strains, resulted in no autoantibody production. These results taken together indicate that excess iodide may well be important in the induction of thyroiditis but that other factors, not least the MHC, must play a crucial role in predisposition to the development of the disease.

Thyroglobulin prepared from CS chickens on either a high or low iodine diet (60 and 13 atoms of iodine/molecule respectively) was assessed for its ability to stimulate the production of autoantibodies. High iodine content Tg stimulated the production of antibodies that bound high iodine content Tg and the thyroid hormones but not low iodine content Tg. Low iodine Tg was a very poor immunogen generating a weak antibody response to high iodine Tg and little or no response to low iodine Tg, T4 and T3. Therefore the CS chicken appears to be tolerant to Tg that has a low iodine content even if it is administered when there are normal levels of iodine in the diet. By contrast obese strain chickens on a normal diet produce anti-Tg autoantibodies which are equally reactive to high or low iodine Tg, on a low iodine diet the levels of autoantibodies to high iodine Tg are reduced but those to low iodine Tg are maintained. Therefore the incidence of thyroiditis and autoantibodies in the OS strain chicken may be unrelated to the intrathyroidal levels of iodine.

B) The BUF rat.

The BUF rat has also been described as a spontaneous model of human Hashimoto's thyroiditis by Silverman and Rose (1971, 1975). The incidence is low in females, 14% in 9-12 week old animals but this rises to about 48% in exbreeder females. This level of thyroiditis can be increased by neonatal thymectomy (NTx) or simultaneous administration of thyroglobulin (Silverman & Rose., 1974, Penhale *et al.*, 1975a, 1976) in conjunction with other substances such as trypan blue (Reuber *et al.*, 1970). It has been demonstrated that the thymectomy model of hypothyroidism does resemble human Hashimoto's disease with respect to the level of AAb produced, infiltration by B and T-cells and the formation of germinal centres. There is also gland enlargement, elevation in the serum TSH level and in the presence of T-cell infiltrates, expression of class II antigens on the thyroid follicular cells (Cohen & Weetman 1987). The thyroid follicular cells expressing class II were also in close proximity to infiltrating lymphocytes; however thyroiditis was observed in two thymectomised animals prior to the expression of class II antigens indicating that class II expression may be indeed secondary to the release of cytokines by lymphoid cells. In the colony described by Cohen and Weetman the incidence of spontaneous thyroiditis was zero; the authors suggested that there may be strain differences between this and the colony described by Silverman and Rose (1974) or that the absence of spontaneous thyroiditis results from differential exposure to environmental pathogens. This points the way to look at the Buffalo strain rat as a potential experimental rather than a spontaneous model. However, unlike the human condition, the thyroiditis observed in the thymectomised BUF rat is only transient; it is not observed within the first five weeks following thymectomy and is diminished by 34 weeks post-thymectomy.

C) The BB rat.

The BB rat although a spontaneous model of diabetes is also useful in the study of autoimmune thyroiditis as it develops thyroid infiltration (on a normal diet) in addition to pancreatic infiltration (Sternthal *et al.*, 1981). Thyroid infiltrating cells are evident from 22-24 weeks of age beginning with the accumulation of dendritic cells and the trapping of T and B-cells; by 24 weeks the cells begin to form small aggregates and subsequently form highly organised lymphoid tissue (Kabel *et al.*, 1987) which is composed of T-cell areas with dendritic cells and high endothelial venules (HEV's), with adjacent B-cell areas containing plasma cells. The structure of this tissue found the BB rat is very similar to mucosal associated lymphoid tissue and to secondary lymphoid organs such as the spleen.

Iodide supplementation of the diet of the BB diabetes prone rat also has an effect on the accompanying thyroid infiltration in the disease Rats supplemented with iodide by the inclusion of potassium iodide in the drinking water from the age of one month exhibited an increase in the incidence of lymphocytic infiltration from 30% to 77% at three months of age (Allen *et al.*, 1987). When this effect is compared to that seen in a normal but genetically similar strain of rat (W-line), iodide supplementation does not induce thyroiditis;

on removal of one lobe of the thyroid of these rats which had the effect of decreasing thyroglobulin reserves and stimulating TSH levels, iodide supplementation increased the levels of thyroid infiltration from 13% to 68%. This study has been complemented by a later study by Mooij *et al.*, (1993b) detailing the immunohistological composition of the thyroid infiltration. An excess of dietary iodine (10X *i.e.* 6.5mg/kg) was shown to accelerate the development of thyroiditis; 50% of animals studies had infiltration by 15-21 weeks the severity of which was also increased. There was only minor evidence of thyroid destruction of follicles at the edge of the lymphoid tissue. This may be due to the level of excess iodine administered; Li *et al.*, (1993) reported that they were able to see evidence of thyroid destruction after 12-15 weeks of a high iodine diet, however this was achieved by the administration of 100X the levels of iodine used in the previous study. The lymphoid structures reported by Mooji *et al.*, (1993b) consist mainly of B-cells surrounded by T-cell areas, it is postulated that these organised lymphoid structures are responsible for the generation of thyroid autoantigen reactive T-cells and for the production of AAbs. The rats that did not go on to develop thyroid associated lymphoid tissue and exhibited a decline in the levels of infiltrating dendritic cells. A similar effect is seen in non-autoimmune Wistar rats that are fed excess dietary iodine; the infiltration in an induced disease is reduced and there is no incidence of anti-colloid autoantibodies (Mooij *et al.*, 1993c). Thus whilst iodine exacerbates an ongoing autoimmune process in the BB rat it may have an effect of 'dampening down' the response in the non-autoimmune Wistar rat.

The mechanism by which dietary iodine exerts its influence over the development of autoimmunity in the rat , chicken and the human is as yet unknown. In the human, iodine has a direct effect on T-cells, B-cells and macrophages. Iodine has been shown to stimulate myeloperoxidase activity in macrophages (Ferguson *et al.*, 1984), increase CD4 and CD8 positive circulating T-cells (Rabinowe *et al.*, 1986) and increase the level of immunoglobulin produced by plasma cells (Weetman *et al.*, 1983). It has also been demonstrated that iodinated proteins are able to increase the maturation of dendritic cells from blood precursors (Kabel *et al.*, 1989, Mooij *et al.*, 1993a); thus iodine may play a role in the development of a critical microenvironment which is conducive to the development of an autoimmune condition. A further role for iodine in enhancing the immunogenicity of thyroglobulin is also a major possibility; excess dietary iodine may allow a high level of iodination of Tg which has been shown to be more antigenic than normal thyroglobulin (Sundick *et al.*, 1987 and de Baets *et al.*, 1987) this may accelerate the development of disease. High levels of iodine also stimulate the thyroid to produce more iodinated proteins so that the presence of iodide may cause autoantigen leakage (Li *et al.*, 1993).

It is clear that these spontaneous models, although they go a long way towards enabling us to analyse the human autoimmune condition; each have their failings which is why it is important to look at the information we can gain from each model to create a global picture. That is also the reason why it is important not to discount experimentally induced models

where it is often easier to delineate the role particular sets of conditions play in the development and maintenance of the autoimmune disease.

1.4.2 Experimental Models of thyroiditis.

Experimental autoimmune thyroiditis (EAT) was first induced in the rabbit by Rose and Witebisky in 1956 (Rose & Witebisky, 1956). The disease was induced by the injection of syngeneic thyroid homogenate emulsified in complete Freund's adjuvant (CFA) and was confirmed by the presence of autoantibodies to thyroglobulin and a mononuclear cell infiltrate in the thyroid gland.

Thyroiditis in rabbits can be induced in rabbits by using 30-100μg of Tg emulsified in CFA injected subcutaneously followed by a further booster 7-15 days later in incomplete Freund's adjuvant. The disease first appears approximately four days following the initial injection and full thyroiditis develops within 3-5 weeks. At about three weeks the maximal titre for anti-thyroglobulin autoantibodies can be detected and the disease remits after approximately six weeks, this is in common with all current experimental models of thyroid autoimmunity, the disease is always remitting.

Experimental autoimmune thyroiditis is induced in the PVG and Wistar rat by a thymectomy and irradiation regime (Penhale *et al.*, 1973). Thymectomy was carried out at weaning at approximately five weeks of age; two weeks after weaning the rats were irradiated with five repeated doses of 200 Rads at two week intervals. It was observed that a lymphocytic infiltration of the thyroid and autoantibodies to thyroglobulin developed spontaneously in 60% of Wistar rats that were thymectomised and whole body irradiated. It was possible to induce thyroiditis in rats that had been whole body irradiated only, but the incidence was reduced to 22%; this was reduced to zero when the irradiation was confined to the thymus region only. In the rats that developed thyroiditis, the histology closely resembled that seen in Hashimoto's thyroiditis; the rats were found to be severely lymphopenic and have reduced mitogenic responses. This was one of the first indications that thymic cells (T-cells) were an important component in the regulation of organ specific autoimmune disease. These rats can be reconstituted with normal T-cells (Penhale *et al.*, 1976) suggesting that the loss of regulatory cells or an excess of effector cells is an important contributory factor in the development of autoimmune thyroiditis. Penhale & Young (1988) have also shown that the normal gut microbial flora of PVG rats is able to affect the development of autoimmune thyroiditis induced by thymectomy. PVG rats maintained in pathogen-free conditions until weaning were significantly less susceptible to the induction of thyroiditis than conventionally reared rats of the same strain. However these rats could be made susceptible again by the administration of the intestinal contents of conventionally reared rats; this effect was transferable to the young of SPF reared rats that were treated in pregnancy with intestinal contents. Therefore the gut flora is able to profoundly influence

the susceptibility to the induction of disease possibly by mimicry between antigens of the gut microflora and the thyroid.

A) MHC restriction.

It was first reported in 1975 by Noel Rose that different inbred rat strains exhibited differing responses in experimental autoimmune thyroiditis as a result of administration of syngeneic thyroid extracts in CFA. Rose found that there was a dissociation in the level of autoantibody production compared to the level of thyroid infiltration observed; this was proposed to be due to two possibilities. Strain related differences in Tg were considered although thyroid extract was shown to be able to inhibit the sera of other (allogenic) rat strains. This indicated that all the antigenic determinants required for induction of disease were present but did not rule out the possibility that those determinants might be differentially expressed in different strains. Results initially suggested that the responses observed were due to allogenic determinants on Tg however responding rats also exhibited specific thyroid infiltration. We now know that thyroglobulin between species is remarkably conserved and that is especially so around the hormonogenic sites.

Whilst antibody formation was not linked to the phenotype of the different rat strains it appeared that the capacity to develop thyroiditis was. Initially the role of genetic influences on the pathology of EAT in the rat remained unresolved but some workers postulated an MHC association with susceptibility (Kotani *et al.*, 1981 and Penhale *et al.*, 1975b) whilst others favoured associations with genes outside the MHC (Lillehoj *et al.*, 1981 and Lillehoj & Rose 1982). The situation was clarified by De Assis-Paiva *et al.*, (1989) who identified that the ability to respond to the induction of EAT in the rat is largely due to the MHC (RT.1) haplotype of that strain. It was demonstrated that animals with the RT.1^{c} haplotype (AUG and PVG^{c}) developed severe disease and that this strongly correlated with antibody titres to Tg; however rat of the RT.1^{u} haplotype (AO and PVG^{u}) only developed a poor level of infiltration despite having good autoantibody levels. These results suggested that these MHC haplotypes were directly associated with high and low responses in the induction of EAT, although the results obtained previously by Lillehoj *et al.*, (1981 and 1982) suggest that there may also be genes outside the MHC or environmental factors which can influence to response to thyroglobulin.

B) Induction of experimental autoimmune thyroiditis (EAT).

It is not just the target organ that plays a role in the genetic susceptibility of the rat to autoimmune thyroiditis; the immune system itself may have an inherent susceptibility. The transplantation of thyroids from high responding rats to low responding rats and vice versa has allowed the dissection of the role the host's immune system plays in the development of disease (Eishi & McCullagh 1988). These workers have used PVG (RT1^{c}) rats which

they indicate are less susceptible to the induction of EAT than DA ($RT1^{avi}$) rats. This is in contrast to the work of De Assis-Paiva *et al.*, (1989) which suggests that these are high responder strains; these rats were crossed to provide PVGxDA F1 hybrids. Allograft tolerance in PVG and DA rats was induced by the administration of F1 bone marrow cells and was tested by the grafting of F1 hybrid skin. Following the establishment of tolerance, PVG rats were grafted with normal DA thyroid tissue into the renal subcapsular region; conversely DA rats were grafted with PVG thyroid tissue into the same region. Grafted rats were then singularly challenged with 0.5 mg rat Tg/CFA and the level of thyroid infiltration and antibody titres were assessed after three weeks; this may account for the differences in the susceptibility of strains as the experiments conducted by De Assis-Paiva *et al.* who used doses of 2mg one week apart, so that the overall dose was much higher. Control PVG grafts in DA hosts, without immunisation by Tg, failed to develop infiltration. In contrast those in immunised hosts developed severe thyroiditis; this was directly comparable to that seen in the hosts own thyroid gland. In DA hosts with PVG grafts, there was no evidence of infiltration in the grafted tissue or in the host tissue. The graft controls of DA onto DA or PVG onto PVG exhibited the expected degrees of infiltration following immunological challenge; the DA strain showed a high degree of infiltration whereas the PVG strain exhibited none.

The results show that thyroid tissue of a resistant phenotype when placed in the appropriate environment of a susceptible phenotype is as vulnerable to EAT induction as susceptible tissue in its host environment. Following on from this, the absence of infiltration in PVG grafted DA hosts excludes the possibility of alloreactivity as a cause of infiltration. In all these results point strongly to a greater role of the immune system in determining the genetic susceptibility to EAT rather than the gland itself. It should be stressed that the graft may play an important role in the production and or presentation of autoantigens, which if they do not result in the induction of the disease may be involved in the perpetuation of the autoimmune condition.

Induction of EAT by immunisation.

Along with the obese strain chicken, high responding mouse strains are the most widely used models of autoimmune thyroiditis. Experimental autoimmune thyroiditis is readily induced using Tg in susceptible mouse strains. A genetic influence on the induction of thyroiditis was first reported in 1965 (McMaster *et al.*,) not long after, thyroiditis was first induced in rabbits. Rose *et al.*, (1971) were able to show that there were distinct strain related differences between CF-1 mice and C3H mice in their susceptibility to induction of autoimmune thyroiditis. CF-1 mice appeared to be particularly susceptible to the induction of disease using homologous thyroid extract in CFA. These workers also compared the effect of different adjuvant preparations in the induction of disease; it was found that differing CFA preparations using identical strains of mycobacterium (H37Ra) were able to give different results, such that with an effective adjuvant they were able to

increase the level of thyroiditis seen in susceptible strain mice (CF-1) and induce thyroiditis in what were previously described as low susceptibility mice (C3H). They were also able to show that cellular immune responses are important in the development of EAT. By injecting Tg intradermally and inducing a DTH response they were able to increase significantly the levels of EAT induced following immunisation with Tg in either adjuvant. It has been shown subsequently that induction of EAT in experimental mouse strains is directly related to the class II MHC or H-2 type that is expressed (Vladutiu and Rose 1971a). H-2^s, H-2^k and H-2^q mice were shown to be excellent responders to thyroglobulin following immunisation as measured by the level of autoantibody production to MTg and mononuclear cell infiltration of the thyroid gland. The H-2^a strain is a moderate responder whereas H-2^b, H-2^d and H-2^v mice are poor responders; the level of thyroid infiltration was shown to correlate well with antibody titres observed. The effect of H-2 type was subsequently shown by Vladutiu and Rose (1975) to be T-cell mediated. Using F1 hybrids between good and poor responders, which were thymectomised and lethally irradiated before reconstitution, they were able to show that only reconstituting cells from good responders transferred responsiveness to Tg induction of thyroiditis in these hybrids. B-cells were unable to transfer responsiveness to thyroglobulin whether they were from poor or good responder mice.

Induction of EAT by B-cells and immunoglobulins.

In experimental and spontaneous models of thyroiditis, autoantibodies to Tg were considered to be a likely cause of tissue injury, however experimental autoimmune thyroiditis has been shown to be extensively T-cell mediated. The history of transfer of thyroiditis with immune sera has had poor reproducibility; a number of attempts to induce the disease in this manner have failed (Twarog & Rose 1968, Jankovic *et al.*, 1969). However there have also been reports of successful transfer of disease in rabbits, using large amounts of sera from donors (Nakamura and Weigle 1969) and in mice injected with pooled immune sera from mice immunised with thyroid extract (Vladutiu & Rose 1971b). Mice were injected with 1ml of pooled sera and after two weeks, thyroid infiltration was observed in the recipients however there was no antibody detected after this time. This effect has recently been repeated in rabbits by the *in situ* perfusion of thyroids with homologous immune sera (Inoue *et al.*, 1993), this enabled the authors to achieve a high concentration of antibody in the gland rather than it being diluted in the periphery. The thyroiditis induced by immune sera was characterised by infiltration of the thyroid, destruction of the follicular structure and granular deposits of IgG and C3 on the basal laminae. The authors were unable to exclude the possibility of immune complexes in the immune sera which may have lodged in thyroid tissue causing tissue lesions. Granular deposits of IgG and C3 in the basal laminae of thyroid follicles have been noted in other successful experiments showing serum transfer of EAT in mice (Tomazic and Rose 1975), immune complexes have also been described in the Obese strain chicken (Katz *et al.*, 1981, Wick and Graf 1972, Kofler *et al.*, 1983) and in induced EAT in mice (Clagett *et al.*, 1974a).

The deposition of immune complexes have also been described in human Graves' disease and Hashimoto's thyroiditis (Werner *et al.,* 1972, Kalderon & Bogaars 1977, Weetman *et al.,* 1989b). There has been a lack of consistent correlation between antibody titres and the extent of thyroiditis, this may be associated with the participation of autoantibodies in immune complex formation rather than a direct interaction with structural components of thyroid tissue. It has been reported that the formation of immune complexes does not coincide with the highest titres of antibodies that are detectable in serum in thyroiditis (Germuth 1953, Dixon *et al.,* 1958), this is due to the fact that pathogenic immune complexes form at near antibody-antigen equivalence thus when immune-complex mediated damage occurs, the relevant antibodies are not detectable in the circulation or are at sub-maximal titres. Thus although EAT can be induced by immune complex deposition it should be stressed that it is not a major factor in the development of experimental or spontaneous disease.

The role of complement components in EAT.

The role of the complement components in the induction of EAT has been investigated using a line of rabbits which are deficient in the sixth complement component C6 (C6-D line), this line is of interest as it has previously been reported that the terminal complement complex (TCC) is deposited in thyroids of patients with Hashimoto's thyroiditis (Jansson *et al.,* 1984b). Normal rabbits when immunised with thyroid extract containing Tg exhibited severe thyroiditis with the degree of infiltration occupying 50-90% of the thyroid, in contrast the percentage of infiltration in the C6 deficient rabbits was less severe, (1-35%). In both cases the infiltrate consisted of mononuclear cells and granulocytes, in the normal rabbits the infiltration was diffuse and extensive this was in contrast to the C6-D rabbits where the infiltration was focal and minimal. Both types of rabbits exhibited extensive deposits of IgG and C3 on the basal laminae, however the normal rabbits also showed deposition of C6 and the membrane attack complex; therefore the terminal complement complex seems to be essential for the induction of severe lesions in the thyroid of rabbits. However thyroiditis can develop in the absence of this complex as shown here in the rabbit and previously shown in the mouse (Nakamura & Weigle 1968). Full sensitivity to EAT induction was restored in C6-D rabbits by the administration of serum from normal rabbits prior to the induction of EAT, administration of C6-D serum to C6-D rabbits had no effect, the C6-D rabbits had normal T-cell function as induction of EAE in these rabbits was comparable to that obtained in normals. In the induction of EAT, it is probable that, the rabbits $CD4^+$ cells become activated and proliferate in response to antigen, helping B-cells produce autoantibodies. This enables the formation of immune complexes at the target organ, which allows the recruitment of granulocytes and cytotoxic T-cells to the site; with the TCC contributing to but not being essential for, the development of EAT. Therefore, although this suggests a role for autoantibody in the development of the disease in rabbits, it does not preclude the essential nature of the T-cell in the initiation of the disease.

Whilst autoantibodies may be involved in the mediation of cell damage in the thyroid, the autoantibodies are not essential for the induction of EAT. AKR mice chronically treated from birth with anti-IgM antibodies to inhibit B-cell development, prior to immunisation with MTg/CFA fail to make detectable anti-Tg autoantibodies. Following immunisation, 63% of those so treated still developed thyroid lesions, this is favourable development of EAT when compared to the 88% incidence in mice treated with normal γ-globulin, control mice also developed good autoantibody responses (Vladutiu 1989). The depression of B-cell numbers in IgM treated mice may have affected their ability to present antigen to Tg reactive T-cells. It is known that Tg specific B-cells exist in mice (Clagett and Weigle, 1974b) and in man (Bankhurst *et al.*, 1973) and that these B-cells are highly efficient presenters of thyroid antigen to T-cells (Hutchings *et al.*, 1987). Therefore, alteration of these cell numbers may have a profound effect on the ability of mice to develop thyroid lesions, not by the reduction of anti-Tg autoantibodies but by a reduced level of presentation of thyroidal autoantigens. Conversely, the generation of an antibody response requires the presence of $CD4^+$ T-cells as shown in the reconstitution of the SCID mouse with human PBMC, depletion of $CD4^+$ T-cells prevents generation of an IgG antibody response (Macht *et al.*, 1993). This is further illustrated by the fact that blockade of $CD4^+$ cells with non-depleting anti-CD4 antibody seems not to affect the development of an anti-Tg autoantibody response (Hutchings *et al.*, 1993). At this point in time there does not seem to be any evidence that non-depleting anti-CD4 treated cells are able or unable to produce cytokines that may be involved in the generation of antibody responses in the absence of effector T-cell responses.

C) Characterisation of the thyroid infiltrate.

Identification of the infiltrating cell phenotypes.

The phenotype of the MTg reactive cells has been shown to be Thy-1, Lyt-1 positive by the *in vivo* administration of appropriate antisera (Simon *et al.*, 1985). Thy-1.2 and Lyt-1.1 antibodies abolished the proliferative capacity of MTg immunised LNC to MTg and PPD (purified protein derivative); similarly antibodies to $H\text{-}2A^k$ were able to block proliferation to MTg and PPD, antibodies to $H\text{-}2A^d$ did not inhibit proliferation. This correlates with the situation seen in the thyroid gland itself in that by day 13 following immunisation, $Lyt\text{-}1^+$ cells predominate over $Lyt\text{-}2^+$ cells but that by day 21 the level of $Lyt\text{-}2^+$ cells gradually increases (Creemers *et al.*, 1984). The kinetic analysis of thyroid infiltration was further characterised by Conaway *et al.*, (1989); following MTg immunisation in LPS *i.v.*, the thyroid was examined immunohistochemically for the presence of $CD4^+$ (CD4), $Lyt\text{-}2^+$ (CD8) *k* light chain (Ig^+ B cell) or $F4/80^+$ (macrophage) cells. The results were consistent with the view that an initial influx of $CD4^+$ cells into the thyroid is responsible for the recruitment of $CD8^+$ cells and macrophages.

Adoptive transfer of LNC has been demonstrated in a number of animal systems such as

the rabbit (Nakamura & Weigle 1967), rat (Jankovic *et al.*, 1969, Twarog *et al.*, 1970) and guinea pig (McMaster & Lerner 1965). Lymph node cells and spleen cells from MTg immunised donors are capable of transferring the disease into syngeneic naive recipients following *in vitro* activation with the non-specific stimulator, Con A (Okayasu 1985). Cells that were cultured *in vitro* with medium alone or LPS failed to transfer disease following *i.p.* injection of approximately $5x10^7$ lymph node or spleen cells. Most of the proliferating cells in either the lymph node or spleen cell cultures were shown to be Thy-1.2$^+$ (T-cells) and it was also shown that depletion of Thy-1.2 or Lyt-1.1$^+$(CD5) cells prior to transfer of *in vitro* activated cells abrogated transfer of thyroiditis. Therefore the cells responsible for the transfer of EAT by definition are T-cells; that B-cell transfer was not responsible for the transfer was confirmed by the lack of reactivity of the recipient serum to normal thyroids. Okayasu *et al.*, (1985) was also able to show that the development of EAT in recipient mice was very rapid and that thyroid infiltration appeared as early as day 8 following transfer and remained in the gland at least 21 days following transfer.

The relative contribution of cells in the thyroid infiltrate has been assessed by using monoclonal antibodies to the CD4 and CD8 epitopes on T-cells which cause depletion of these cells. Kong *et al.*, (1989b) were able to show that removal of CD4$^+$ T-cells by injection of depleting anti-CD4 on days minus one and six prior to MTg immunisation on days 0 and 7, followed by a single injection on day 13, abrogated development of disease as shown by infiltration and the development of an autoantibody response. In contrast, anti-CD8 had little effect on the induction of disease; all recipients had infiltrated thyroids but the level of infiltration was reduced, antibody titre was unaffected. Anti-CD4 was also inhibited an ongoing response when administered seven days after immunisation whereas anti-CD8 had no effect. On advanced EAT the effect was slightly different; anti CD4 was able reduce infiltration to a greater extent than CD8 alone, but a combination of anti-CD4 and anti-CD8 therapy was able to reduce the degree of infiltration in 50% of animals and clear the infiltration in the remaining animals by day 28 following immunisation. This indicated that the CD8 cells, although not important for the initiation of the disease, may be very important in the maintenance of the infiltration; the fact that depletion of CD8 cells did not enhance thyroiditis or antibody levels suggests that any cells that may be exhibiting a regulatory control in EAT are not CD8+ suppressor cells. The protective effect of these monoclonal antibodies was shown to be long lasting as at 70d post-antigenic challenge and 45d since the last anti-CD4 administration, the level of thyroid infiltration was very low; this effect was greatest when anti-CD4 and anti-CD8 were used in conjunction with each other. Autoantibody expression (anti-Tg) increases over this period, thus despite a continuous antigenic stimulus a chronic inflammation was not able to develop once the initial infiltration was cleared. This lack of infiltration was not due to a depletion of T-cells in the periphery as by day 70 a high enough level of T-cells were detected in the periphery for recruitment into the thyroid, this degree of recovery by day 70 is sufficient for EAT to be induced following peripheral deletion by monoclonal antibody therapy

(Kong *et al.*, 1989a). The composition of the infiltrating cells in control mice was similar to that found by Conaway *et al.*, (1989), from day 21, 30-35% of the infiltrate was comprised of T-cells, <6% B-cells and a small number of polymorphonuclear cells, remaining cells were shown to be macrophages.

This composition is repeated following the transfer of MTg *of vitro* activated spleen cells (Conway *et al.*, 1990), T-cells accounted for approximately 35% of the infiltrating cells at day 7 following transfer, increasing to 56% by day 10, the ratio of CD4 and CD8 cells also changed in that the proportion of CD8 cells increased with time following transfer. As in the previous work (Conaway *et al.*, 1989) the relative proportions of CD8 cells increased over time, these cells may indeed be responsible for the cytotoxic activity that has been reported *in vitro* (Creemers *et al.*, 1983) and seen in T-cell clones from Hashimoto's patients (Bagnasco *et al.*, 1987, Mackenzie & Davies., 1987), once again it is postulated that CD8 cells are involved in the latter stages of the disease.

T-cell lines and clones involved in EAT.

The role that T-cells play in the development of EAT have been further characterised by the development of lines and clones specific to thyroid antigens. T-cell clones obtained from high responder ($H\text{-}2^k$) mice, which are known to proliferate to MTg, have been shown to be capable of transferring autoimmune thyroiditis into irradiated naive syngeneic recipients (Weigle *et al.*, 1987). These cells have been shown to be of the $CD4^+$ phenotype and capable of responding to thyroid extract, pig Tg, bovine Tg as well as mouse thyroid extract and are therefore not restricted to species specific determinants. This cross reactivity has also been demonstrated for two T-cell lines developed by Champion *et al.*, (1984) that are reactive against native mouse Tg, also rat and human Tg. These T-cell lines are also capable to transferring EAT into naive irradiated recipients (Champion unpublished data) although reproducibility of this effect is not high. These cells have been further characterised by the establishment of Tg-reactive T-cell hybridomas and have been used extensively in this thesis to delineate the specificity of the antigen response to Tg. Maron *et al.*, (1983) have also been able to induce EAT using an MTg specific T-cell line (TF1) in irradiated recipients or athymic nude mice. This same T-cell line when attenuated by irradiation has been shown to be capable of preventing the subsequent induction of EAT by immunisation with Tg/CFA or by the transfer of intact activated cell TF1 cells. Therefore a T-cell line is able to vaccinate against the induction of autoimmunity with fresh antigen or primed cells, but this is unable to modify the level of anti-Tg autoantibody production seen in these mice.

D) Prevention of EAT.

The induction of tolerance in EAT.

Much work by many people has elucidated the fine specificity of the T-cell involvement in experimental autoimmune thyroiditis. In a normal non-autoimmune situation, autoreactive T-cells in the periphery are held in a state of tolerance by peripheral tolerance mechanisms such as anergy, suppression or deletion. Autoreactive T-cells, although circulating may be held in a tolerant state. However it has already been shown that this tolerance may be broken by lethal irradiation and immunisation of reconstituting cells. It has also been shown that repeated high doses of antigen in the absence of adjuvant are able to over-ride the normal regulation within the system. Elrehewy *et al.*, (1981) showed that repeated injections of syngeneic thyroglobulin into good responder mice were able to induce thyroid infiltration and a good accompanying autoantibody response whereas in poor responder mice, syngeneic Tg was only able to induce a low antibody response with no thyroid infiltration. Thus autoreactive T-cells do circulate but are held in a non-responsive state by tolerance mechanisms.

It has been reported that spleen cells from MTg tolerised animals are able to transfer that tolerance to syngeneic mice challenged with Tg (Kong *et al.*, 1982). This tolerance has been shown to be T-cell mediated as it may be abrogated by the administration of anti-Thy-1 (murine T-cell marker). Although the authors suggest this as evidence for suppressor T-cells it is difficult to determine whether the suppression would be mediated by T-cells directly or by T-cell products; what is clear is that T-cells play a pivotal role in the disease process being essential for autorecognition and perpetuation of the immune response and also the regulation of that specific immune response. It appears that the difference between susceptible strain mice and non-susceptible stain mice is a fine dividing line, non-responder mice can be converted to responsive mice by the administration of 100mg/kg of cyclophosphamide (Cy) prior to administration of thyroglobulin/CFA (Vladutiu, 1982) as measured by the degree of infiltration within the thyroid. The administration of Cy also had the effect of increasing the degree of infiltration of the thyroids in high responder mice although the differential increase in induction was not as great in these as was seen in low responder mice. In these experiments the administration of Cy had the effect of reducing the autoantibody levels at high doses (100 mg/kg) but increasing them at low doses (20 mg/kg). The magnitude of thyroid lesions has often been shown not to correlate with the titre of anti-Tg antibodies (Vladuitu & Rose 1972), therefore the authors suggest from this study that Tg antibody production and the development of thyroid infiltration are influenced by separate control mechanisms. Further to these studies showing the importance of MHC, in particular, the class II sub-region , it has been shown that the D-end genes are able to reduce the level of thyroid infiltration without influencing the levels of autoantibody production (Beisel *et al.*, 1982). These findings further support the lack of correlation between the levels of autoantibody production and thyroid infiltration

(Vladuitu & Rose 1972, Rose *et al.,* 1981, Elrhewy *et al.,* 1981). Vladutiu (1982) also suggests that low-responder strains have a suppressor activity which is not present or may not be present to such a degree in high-responder strains, that inhibits the development of autoimmune thyroiditis even after administration of exogenous autoantigen.

Tolerance may be induced by the administration of tolerogenic doses of MTg (100-200 μg, 2 doses) but also by the administration of TSH (0.25IU/day) following which peak endogenous levels of MTg are achieved after 3 days (Kong *et al.,* 1989a). By the administration of paired monoclonal antibodies to distinct epitopes on the L3T4 (CD4) or Lyt-2 (CD8) molecules, Kong *et al.,* (1989a) demonstrated that the cells mediating resistance in the MTg pre-treatment and TSH infusion were of the CD4+ phenotype; and were also insensitive to cyclophosphamide. Using the protocol established by Kong *et al.,* (1989a) our own laboratory had been able to determine that suppression of antibody formation also resides in the CD4+ subset (Parish *et al.,* 1988).

Further to this study it has been shown that the level of tolerance induced by the administration of high levels of MTg is correlated with the length of time that MTg levels are raised above the normal range (Lewis *et al.,* 1992). Peripheral Tg levels must be elevated for a critical period of 2-3 days within a ten day period prior to challenge with MTg; this can be enhanced by the administration of LPS prior to tolerisation which may have the effect of altering the kinetics of MTg clearance from the circulation, thus allowing thyroglobulin levels to remain elevated for a longer period of time. The initial clearance rate (α phase) has a $T_{1/2}$ of 3h, the long term clearance rate (β phase) exhibits a Tg $T_{1/2}$ of approximately 10 h (Lewis *et al.,* 1987), LPS has the effect of lengthening the initial α phase to 5 h and in doing so is able to prevent the induction of EAT. The overall conclusion is that the higher the level of circulating Tg over a critical period of time, the greater the level of tolerance induction to Tg; the effect of LPS is to prolong the half life of circulating Tg such that the tolerising effect is comparable to higher doses of Tg. This resistance to EAT has again been shown to be mediated by the CD4+ subset of T-cells (Lewis *et al.,* 1992) and has been shown to be long lasting (up to 73 days) following the tolerising regime (Fuller *et al.,* 1993). The authors suggest that circulatory Tg may serve a physiological role in maintaining normal self tolerance by the sustaining of low levels of suppressor T-cell (Ts) cell activation.

There is further evidence for the existence of suppressor cell regulation of the induction of EAT. It has been demonstrated that transfer of EAT is augmented by the irradiation of susceptible recipient mice prior to transfer of MTg *in vitro* activated spleen cells (Williams *et al.,* 1987); in contrast this is not achievable by the transfer of *in vitro* activated spleen cells from non-susceptible strains such as BALB/c. This enhancing effect can be reversed by the transfer of naive spleen cells to an irradiated recipient prior to the transfer of activated cells; the removal of T-cells from the naive population reduced, but did not completely

eliminate the ability of these cells to suppress activated cells. These results confirm that natural suppressor cells seem to exist in the naive animal prior to the administration of exogenous antigen and that they can function after initial sensitisation of effector cells and therefore regulate EAT at the effector stage; if this function of suppressor cells could be harnessed in ongoing autoimmunity then it holds great potential as possible therapy. In the human, most cases of autoimmunity are only presented once the disease has become established; therapy at this stage may prevent further destruction and may allow the regeneration of destroyed tissue.

Thyroiditis can also be induced by MTg in lipopolysaccharide (Esquivil *et al.,* 1977). Simon *et al.,* (1985) have shown that lymph node cells (LNC) taken from MTg immunised animals are able to proliferate *in vitro* to MTg and that this proliferation could be consistently enhanced by the addition of equal numbers of syngeneic, irradiated spleen cells. It has been reported in another autoimmune system that the proliferative responses to myelin basic protein (MBP) were only detectable on removal of adherent cells from the primed LNC population and the adding back normal spleen cells (Ben-Nun & Lando 1983), the reasoning behind this being that it may increase the number of APC's available for the presentation of the autoantigen and that it may help overcome the action of suppressor cells which may be in the primed LNC population. However Simon *et al.,* (1985) found no substantial benefit in the removal of adherent cells on MTg specific proliferation.

Prevention of EAT by anti-CD4 monoclonal antibody.

Hutchings *et al.,* (1993) have shown that treatment of MTg primed effector cells with anti-CD4 prior to the *in vitro* activation and subsequent transfer of those cells to naive recipients was able to abrogate the development of EAT. Non-depleting anti-CD4 MAb given to donors immediately prior to sacrifice or during *in vitro* culture failed to prevent transfer of disease, whereas, a depleting antibody given prior to sacrifice was able to abrogate the transfer of disease and depleting anti-CD8 could not; this indicated the pivotal involvement of $CD4^+$ T-cells in the transfer of disease. The mechanism of anti-CD4 mediated suppression appeared to be by unresponsiveness rather than receptor blockade as non-depleting anti-CD4 had no effect in culture or immediately prior to sacrifice. The suppressive effect on the CD4 treated cells was transferable to naive recipients; transfer of cells treated this way specifically prevented the induction of EAT in naive donor mice by the administration of MTg/CFA following transfer; however there was no significant effect on the level of anti-MTg autoantibody responses. In the same experiment it was shown that the suppression of the response was specific to MTg responding cells; as anti-SRBC antibody and anti-OVA proliferative responses remained intact. It is postulated that anti-CD4 may have a differential effect on Th1 cells than Th2 cells; Th1 cells may become permanently modified and Th2 cells just temporarily blocked, therefore thyroiditis and antibody responses would be abrogated in animals directly treated with antibody. Following transfer, Th2 cells may emerge from receptor blockade whilst Th1 cells were still modified,

the dominance of the Th2 subset would mean that normal antibody responses would be mounted in the absence of thyroiditis whilst the Th1 subset was still resistant to antigenic challenge.

Prevention of EAT by immunosuppressive agents.

The prevention of the induction of EAT is not confined to the administration of antibodies to T-cell markers. Classical imunosuppressants such as cyclosporin A (CsA) and FK506 are potent inhibitors of $CD4^+$ T-cell activation and production of IL-2, IFN-γ and other cytokines (Schreiber and Crabtree 1992). FK506 is more potent than CsA and has been shown to be efficacious in a number of autoimmune diseases such as diabetes (Miyagawa *et al.*, 1990) and EAE (Inamura *et al.*, 1988) and has now been shown to be effective in the suppression of thyroiditis in irradiated Tx PVG/c rats (Tamura *et al.*, 1993). Administration of FK506 was shown to reduce the incidence of infiltrating T-cells (CD4 and CD8) and the level of expression of CD18 (LFA-1), CD54 (ICAM-1) and MHC class II on T-cells, endothelial cells and thyrocytes. The level of FK506 achieved *in vivo* was high enough to prevent the production of cytokines by $CD4^+$ Th1 cells whilst sparing Th2 cell production of IL-10; there does not seem to be any development of a more severe form of granulomatous thyroiditis as seen in the mouse as a result of inhibition of the Th1 response. However, this model does not rely on the use of autoantigen to induce the disease but on the removal of the regulatory mechanisms involved in the susceptible autoimmune state, this may account for the fact that a Th2 population is not expanded by inhibiting the Th1 population in the absence of antigen.

Prevention of EAT by T-cell vaccination

EAT induction may be prevented by the vaccination of mice prior to induction of EAT with a Tg specific T-cell line; this is achieved by the attenuation of these T-cell lines by irradiation or mitomycin C treatment (Maron *et al.*, 1983). Supporting this, Bedin *et al.*, (1993) have shown that immunisation of CBA/J mice with cytotoxic T-cell hybridomas specific to a peptide fragment of porcine Tg are also able to prevent subsequent induction of EAT in these mice. The authors have previously shown that EAT protection can also be induced by antibodies against the TCR of the cytotoxic hybridoma (Texier *et al.*, 1992a); therefore it is speculated that immunisation with a cytotoxic hybridoma may lead to the generation of anti-idiotype autoantibodies to the TCR and the resulting id-anti-id interactions may be involved in the modulation of disease.

Prevention of EAT by modulation of adhesion molecule function.

Adhesion molecules have an important role to play in inflammatory and the immune responses, by initiating and strengthening T-cell/target interactions. The role that these types of molecules may play in the development of an autoimmune disease such as thyroiditis has been investigated by Metcalfe *et al.*, (1993). The ICAM-1/LFA-1 ligand interaction has been shown to be involved in T-cell proliferation (Dougherty *et al.*, 1988),

cytotoxicity (Makgoba *et al.*, 1988) and adhesion to endothelial cells (Springer *et al.*, 1987). These adhesion molecules are expressed on thyroid epithelial cells isolated from Graves' and Hashimoto's patients (Bagnasco *et al.*, 1991 and Weetman *et al.*, 1989a), further to this ICAM-1 can also be expressed on cultured thyroid cells (Weetman *et al.*, 1990a & Tandon *et al.*, 1991). It is likely that any modification of these types of interactions in EAT would have an effect on the development of disease given the central role that T-cell and thyroid cells play in the disease process. Metcalf *et al.*, (1993) have shown that anti-LFA-1 and ICAM-1 antibodies were able to reduce the levels thyroid infiltration and anti-ICAM-1 antibody reduced the level of autoantibody production in rats following the induction of EAT. Administration of these antibodies inhibited the proliferation of spleen cells in response to autoantigen; however anti-ICAM-1 antibody was unable to inhibit the cytotoxicity of spleen cells to thyroid epithelial cells. Given the effect that these two monoclonal antibodies have on the proliferation of cells in response to antigen; the authors speculate that the antibodies may be having their effect at the level of antigen presentation to T-cells by APC's. The effect may be on the initiation of the immune response or be acting at the level of infiltration of the gland and preventing the extravasation of reactive cells from the blood into the tissues, in particular the thyroid tissue. The authors also speculate that other ligands for LFA-1 such as ICAM-2 & 3 may be important in the production of autoantibodies, which may account for the lack of effect anti-ICAM-1 had on the level of autoantibody production.

Prevention of EAT by modulation of cytokine function.

The cytokines, interleukin-1 and TNF-α are believed to contribute to the inflammatory responses associated with autoimmune diseases; for example enhanced IL-1 production has been reported to be associated with increased disease severity in rheumatoid arthritis (Shore *et al.*, 1986) and the rheumatoid synovium has been shown to contain GMCSF, IFN-γ and TNF-α (Xu *et al.*, 1989 & Hopkins and Meager 1988). Recent studies have shown that TGF-β has immunoregulatory effects on T and B-cell proliferation, cytokine production and immunoglobulin production; also that TGF-β knockout mice develop massive systemic autoimmunity and organ destruction (Shull *et al.*, 1992), confirming a role for this cytokine in immunoregulation. The *in vivo* effects of TGF-β are demonstrated by the co-administration of r-TGF-β during the induction of collagen arthritis; it is able to suppress the severity and incidence of arthritis in DBA/1 mice, it is also able to suppress the development of relapsing EAE in SJL mice (Kuruvilla *et al.*, 1991). In the human, TGF-β has been shown to decrease the proliferation of PBMC's from Graves' patients and normal controls in response to polyclonal stimuli and of a thyroid derived T-cell to IL-2 (Widder *et al.*, 1991). TGF-β also inhibits the response of thyroid derived T-cells to autologous thyroid epithelial cells and decreased the expression of TPO and HLA-DR in these cells, therefore TGF-β may to act as a counter-regulatory cytokine in Graves' disease as it is produced by thyroid infiltrating lymphocytes and the target thyroid epithelial cells. This cytokine may be important in the suppression of disease in those individuals that are

predisposed to the development of autoimmunity; the effect of this cytokine may be lost following massive immunostimulation in the acute stage of the disease, conversely this cytokine may play an important role in the remission of disease. TGF-β is secreted in an inactive form and it may be that conversion into the active form may be defective in those individuals that are susceptible to the development of disease.

Clearly the course of the disease in EAT is multifaceted and it has been shown by many workers that modulation of the disease may be achieved by a number of means. The blockade of the responsiveness of the T-cell, in particular the $CD4^+$ T-cell profoundly inhibits the course of the disease also the blockade of the essential interactions involved in the initiation of an immune response are also able to suppress the level of disease. T-cells products are also implicated in the regulation of EAT, although a precise role for particular cytokines remains as yet undefined. It appears that EAT in common with many diseases known to man, has multiple components which are all important in the development of disease but that no single component can be deemed to be uniquely responsible for the development of EAT; they all work together to constitute a very interesting set of predisposition's which allow the condition to develop.

1.5 The role of thyroid epithelial cells in EAT.

A) Interferon-gamma induction of class II and modulation of EAT.

In recent years a number of different methods have been used for the induction of thyroiditis which differ quite considerably from established methods but that still depend on T-cells. The role that cytokines play in the induction and development of EAT has been the subject of intense speculation and scrutiny. Hanafusa *et al.*, (1983) first reported the aberrant expression of MHC class II molecules on thyrocytes from Graves' and Hashimoto's patients. Interferon-γ induces the expression of MHC class II molecules on cultured thyrocytes (Todd *et al.*, 1985) and administration of IFN-γ increases the severity of several autoimmune diseases (Mauritz *et al.*, 1988, Campbell *et al.*, 1988, Engleman *et al.*, 1981, Jacob *et al.*, 1989, Cooper *et al.*, 1988). In parallel to this, neutralisation of IFN-γ by anti-IFN-γ antibody delays the onset and reduce the severity of murine SLE (Jacob *et al.*, 1987).

Kawakami *et al.*, (1990) have shown that the administration of $3x10^5$ units of IFN-γ per day for a total of five weeks was able to induce thyroiditis in C3H ($H\text{-}2^k$) mice. The expression of class II antigens on epithelial and thyrocyte cells was detectable by four days following the first injection and continued throughout the course of treatment. Over this time period, serum T3 and T4 concentrations were significantly decreased although not to the same extent as in EAT conventionally induced by Tg. Anti-Tg autoantibodies were also detectable following treatment of one week becoming maximal by four weeks but at all times remained below that obtained in conventionally induced EAT, anti-microsomal

antibodies were only detected in 4% of treated mice. The percentage of mice that developed a lymphocytic infiltration was 16% but only four weeks after the first administration of cytokine, thus the class II expression in thyrocytes was not able to induce full blown thyroiditis or all of the functional changes associated with the condition, but was able to induce a partial thyroiditis. In a complementary set of experiments, Tang *et al.*, (1993) have shown that the treatment of mice with anti IFN-γ following the induction of EAT is able to reduce the level of infiltration within the thyroid gland in particular the level of $CD8^+$ T-cells which are responsible for the destruction of the thyroid and also reduce the level of autoantibodies produced. This role of IFN-γ is supported by the fact that direct injection of IFN-γ into the thyroid of susceptible mice results in the induction of EAT and suggests a strong pathogenic role for IFN-γ (Remy *et al.*, 1987). Other studies have demonstrated that IFN-γ can suppress EAE (Voorthuis *et al.*, 1990) and EAT (Vladutiu and Sulkowski 1980) and that antibodies to IFN-γ may increase the severity of disease (Billiau *et al.*, 1988 & Wiesenberg *et al.*, 1989), therefore it has been postulated that IFN-γ may play an important role in the development and or maintenance of the autoimmune condition.

This data from the mouse model ties in with data obtained in Chinese patients being treated with rh-IFN-γ for chronic hepatitis B (Kung *et al.*, 1990). Following treatment with IFN-γ there was an increase in the number of circulating lymphocytes and in particular the number of DR positive T-cells with a concomitant decrease in the number of granulocytes and IL-2R positive cells, the elevation in DR positive cells was maintained up to 38 weeks after the discontinuation of therapy. Fifty percent of patients developed autoantibodies after therapy but none developed any autoantibodies associated with thyroiditis, these patients were subsequently typed and none were found to be of a DR type associated with development of thyroiditis in Chinese populations (HLA-DRw9). In an earlier study by Burman *et al.*, (1986) using IFN-α therapy it was found that development of thyroid dysfunction occurred in those patients that already had circulating thyroid autoantibodies before treatment. Putting these two sets of data together suggests that in the human, susceptibility to disease is the key to the development of disease following therapy with IFN-γ, this is what we already know to be true in the mouse given the importance of H-2 haplotype in the induction and development of disease. This result is important in our understanding of the pathogenesis of the human condition of thyroiditis, the sequence of events that lead to thyroid destruction are complex and as yet only beginning to be unravelled by the use of autoimmune models.

In contrast to this protective effect by anti-IFN-γ, the administration of anti-IL-2 receptor antibody following transfer of MTg activated spleen cells in susceptible mice has a profound effect on the level of disease severity (Braley-Mullen *et al.*, 1991). EAT induced in the presence of anti IL-2 receptor is more severe and is characterised by extensive infiltration, follicular destruction and granuloma formation. Recipients also had higher autoantibody

responses than observed in normal conditions of EAT induction; these increases were not limited to particular subclasses, all were represented as normal. Transfer of this granulomatous thyroiditis required the presence of anti IL-2 antibody within the first 72 h of culture, and was shown to be mediated by $CD4^+$ cells which is in common with EAT induced by the conventional method. These results might be explained by the activation of different T-cell subsets in the presence or absence of anti IL-2 receptor, Th1 clones have been shown to produce autocrine growth factor IL-2 and IFN-γ whilst the Th2 subset produces autocrine growth factor IL-4 in addition to IL-5 &6 (Mosman & Coffman 1989 & Janeway *et al.*, 1988). Th2 cells have been shown to be effective helper cells for B-cells immunoglobulin production (Stevens *et al.*, 1988, Snapper & Paul 1987), whilst Th1 cells are much less effective. Thus it is possible that the granulomatous thyroiditis is mediated by Th2 cells whilst the cells involved in the induction of normal EAT are of the Th1 phenotype; this may be due to the direct induction of a less severe form of the disease or suppression of the activation of Th2 effector cells. There may be preferential binding of anti IL-2 receptor to the Th1 subset allowing the inhibition of activation of Th2 cells and response to locally produced autocrine IL-4 growth factor. This is supported by the finding that *in vivo* injection of anti IL-2 receptor is able to suppress the function of Th1 cells whilst having little effect on Th2 cells (Diamantstein *et al.*, 1988) and that IL-4 producing clones have been shown to be capable of causing EAT (Romball and Weigle 1988). Further to this, it has now been shown by the some workers, (Stull *et al.*, 1992), that anti-IFN-γ antibody is also able to induce granulomatous thyroiditis; this result is consistent with the hypothesis that Th2 cells are responsible for the induction of granulomatous thyroiditis. Interferon-γ is produced by Th1 cells (Mosmann *et al.*, 1986) which can inhibit the growth of Th2 cells (Gajewski and Fitch 1988); inhibition of the effect of IFN-γ would allow the expansion of a Th2 subset. In addition to this finding, the authors also report that activation of effector cells *in vitro* with MTg in the presence of anti-IL-4 can result in a decreased severity of lymphocytic infiltration and the lowering of anti-Tg autoantibody responses.

A T-cell clone has now been established which is derived from a low responder, $H\text{-}2^b$ mouse, which has a Th2 cytokine profile and is able to transfer EAT following *in vitro* stimulation (Hiyama *et al.*, 1993). On thyroglobulin stimulation the clone produced IL-4 and 6 but not IL-2 or IFN-γ. It is clear that low responder strains have the potential to develop autoimmunity as administration of cyclophosphamide prior to immunisation with Tg is able to convert low responder mice to high responder mice (Vladutiu, 1982). The role that B cells and the autoantibody produced by those cells might play in the induction of EAT, in particular granulomatous EAT has been investigated. Braley-Mullen *et al.*, (1994) have shown that if B-cells are removed prior to the transfer of activated spleen cells which have been cultured with anti-IL-2 receptor antibody, this markedly inhibits the development of granulomatous thyroiditis. In contrast, removal of B-cells did not effect the development of a control lymphocytic thyroiditis.

Thus there are examples where administration of systemic IFN-γ is able to suppress autoimmune disease (Vladutiu and Sulkowski 1980) and others where local administration is exacerbatory (Mauritz *et al.*, 1988, Charreire 1989) it is suggested by Stull *et al.*, (1992) that because exacerbation of EAT occurred when IFN-γ was neutralised during the *in vitro* activation step or early in cell transfer means that IFN-γ itself is actively able to inhibit the effector cells for granulomatous thyroiditis before they are able to traffic to the thyroid. Once in the thyroid, these cells are still susceptible to the effects of interferon, as antibody given late after transfer was able to enhance the level of disease. This suggests that cells homing to the thyroid are sensitive to locally produced IFN-γ such that its presence may serve to limit the level of disease but may also have the detrimental effect of ultimately prolonging the disease into a chronic condition by the up-regulation of class II on the thyroid cells and subsequent presentation of thyroidal autoantigens.

B) The role of the thyroid in development of EAT.

The models of EAT all point to the crucial step of T-cell involvement in disease and speculate on a role for autoantibodies, but perhaps the most important factor in this condition is the role that the thyroid which is the principle target organ plays in the development and perpetuation of the disease. In 1981, Yeni and Charreire showed that lymphoid cells derived from high responder CBA mice could be sensitised to and proliferate in response to syngeneic thyroid monolayers (Yeni *et al.*, 1981) and the that proliferation was T-cell dependent (Charreire 1982) and H-2A sub-region restricted (Salamero and Charreire 1983a). The greatest proliferative responses of lymphocytes to monolayers were observed from strains of mice of high responding haplotypes such as $H\text{-}2^k$ and $H\text{-}2^s$ whereas low responder mice lymphocytes exhibited minimal proliferation to syngeneic TEC's (Salamero and Charreire 1983b). Following sensitisation on thyroid monolayers these T-cells are were able to cause thyroid infiltration following injection into naive recipients (Charreire and Michel-Bechet 1982); cells generated in this way have also been shown to have a cytotoxic capacity against TEC's (Salamero and Charreire 1985). Salamero *et al.*, (1983b) have shown that in cultured thyroid epithelial cells, Class I antigens appear to be located on the apical surface of the TEC whereas Class II antigens are located on the basal surface; the levels of these antigens is higher and more evenly distributed in freshly isolated cells (Salamero *et al.*, 1983c) However following 24-48 h incubation with syngeneic lymphocytes, the apical surface of TEC's can be shown to also express class II antigens (Salamero *et al.*, 1985), thus the surface that bears Tg and class II antigens is in direct contact with lymphocytes.

Class II antigens are involved in the initiation of an immune response to exogenous antigens, the expression of which is normally restricted to professional antigen presenting cells such as B-cells, macrophages and dendritic cells. The potential does exist for non-professional antigen presenting cells to be able to present endogenous antigen in the context of class II,

if expression of class II can be upregulated. The first report of aberrant class II expression on thyroid epithelial cells came in 1983; human thyroid epithelial cells which do not normally express HLA-DR molecules were shown to express class II antigens in autoimmune thyroid disease (Hanafusa *et al.*, 1983 & Jansson *et al.*, 1984b) also that thyroid epithelial cells can be induced to express these antigens following culture with phytohaemagglutinin (PHA), ConA and pokeweed mitogen (PWM) (Pujol-Borrell *et al.*, 1983). Further to the work of Salamero and Charreire (1983b) in the murine system, it has also been shown that human class II^+ thyrocytes are able to present thyroid antigen to human T-cell clones derived from the cellular infiltrates of Graves' disease patients (Londei *et al.*, 1985), they are also able to present flu antigens to specific T-cell clones (Londei *et al.*, 1984). Given that the potential exists for class II expression, the mechanism by which this may relate to the disease process has been extensively studied.

C) The role of inflammatory cytokines in EAT.

It has been shown that IFN-γ is able to induce the expression of class II antigens on murine cultured TEC's (Salamero *et al.*, 1985), in particular this expression is induced on the apical surface of these cells after 24-48 h incubation, IFN-γ can also induce the expression of HLA-DR molecules on human thyroid epithelium (Todd *et al.*, 1985) and this induction is enhanced by TSH (Todd *et al.*, 1987a). The level of class II expression on freshly isolated thyrocytes varies between patients and that the incidence and intensity of expression appears to differ between Hashimoto's patients and Graves' patients (Todd *et al.*, 1987b). Hashimoto's patients and some Graves' patients thyroids showed strong expression of all HLA sub-regions (DR, DP and DQ), in other Graves' patients there was a hierarchy of expression in which DR was most strongly expressed followed by DP and then DQ. The level of class II expression by thyrocytes was located in areas of infiltration and the extent of this infiltration and expression of class II correlated with the proportion of thyrocytes expressing class II, it was also noted that the capillary endothelium expressed HLA-DR, DP and DQ. This pattern of expression is reproduced *in vitro* by the addition of IFN-γ to the culture media of TEC's and enhanced by TSH.

D) Thyroid epithelial cell costimulatory function.

There is conflicting data on whether TEC's are able to present thyroid antigens *in vitro* and hence *in vivo*. An antigen presentation capacity has been demonstrated for thyroid epithelial cells by Remy *et al.*, (1986), these cells have the capacity to present thyroglobulin to syngeneic lymphocytes whereas allogeneic TEC's are unable to induce a response, this presentation to lymphocytes is confined to good responder strains (CBA, $H\text{-}2^k$) as poor responder (C57Blk, $H\text{-}2^b$) TEC's are unable to stimulate high responder lymphocytes. These results contrast with Ebner *et al.*, (1987), who show that using high responder C3H mice they are able to up-regulate class II expression in response to IFN-γ but these cells

are unable to present antigen to Tg primed LNC *in vitro*. This is not due to a lack of IL-1 as the authors show that IL-1 is secreted by the TEC's which is required as a costimulatory factor by Th1 cells. The only differences between these two studies is in the strains studied, it is not known if there are any intrinsic differences between the antigen presentation capacity of these strains. In conjunction with IFN-γ, tumour necrosis factor-alpha (TNF-α) is able to act synergistically to induce class II expression on human and animal thyroid cells (Weetman *et al.*, 1988 & Buscema *et al.*, 1989). IFN-γ is also able to induce TNF-α production (Ruggerio *et al.*, 1986) and the expression of TNF-α receptors (Tsujimoto *et al.*, 1986). Together these cytokines may play an important role in the pathogenesis of thyroid disease.

Thyroid epithelial cells may be able to act as antigen presenting cells, however it remains true that TEC's are not professional APC's and therefore may lack certain costimulatory function molecules. One group of molecules that have been shown to be important in the induction of immune responses are the adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1), CD2, LFA-3 and the intercellular adhesion molecules (ICAM1-3). These adhesion interactions are critical to efficient T-cell activation by the strengthening of the interactions between T-cells and their targets. Cultured thyroid epithelial cells derived from Graves' disease patients have been shown to express ICAM-1; the percentage of and intensity of expression can be increased by IFN-γ, IL-1 and TNF-α. Spatially, ICAM-1 expression can be demonstrated on thyroid cells in the areas of lymphocytic infiltration in both Graves' and Hashimoto's patients (Weetman *et al.*, 1989a, Bagnasco *et al.*, 1991), it is not clear whether the epithelium is able to express class II antigen and ICAM-1 as a response of local cytokine synthesis by infiltrating cells or whether this is causative of the infiltration. It has been reported by Hamilton *et al.*, (1991) that most of the class II expression by the thyroid cells is the result of local release of IFN-γ. There is good localisation of class II expression with lymphocytes in addition to this focal thyroiditis tends to occur as lymphoid aggregates which can form germinal centres, these aggregates have been shown to contain many IFN-γ positive lymphocytes with a region of class II positive thyrocytes surrounding them, probably as a result of cytokine diffusion.

In Graves' ophthalmopathy, where the target organ is the orbital tissue, it has been shown that this tissue expresses ICAM-1, endothelial adhesion molecule-1 (ELAM-1), vascular adhesion molecule-1 (VCAM-1) and LFA-3 (Heufelder and Bahn 1993), LFA-3 and ICAM-2 have also been shown to be expressed on human thyroid epithelial cells (Tandon *et al.*, 1992) which can be upregulated by IL-1 but not by TSH. Ebner *et al.*, (1987) have demonstrated that thyrocytes secrete IL-1 and it has been suggested that IL-1 may regulate thyroid cell proliferation (Kawabe *et al.*, 1989). It has also been shown to inhibit thyroid peroxidase gene expression (Ashizawa *et al.*, 1989) and also acts as a local inflammatory mediator and is able to induce T and B-cell proliferation. TEC's have also been shown to

produce TNF-α (Zheng *et al.*, 1992) which can be produced in response to IFN-γ (Ruggerio *et al.*, (1986) which may serve to potentiate class II expression. Thyrocytes have also been shown to produce IL-6 which plays a role in B-cell differentiation and T-cell activation (Weetman *et al.*, 1990b), IL-6 also inhibits thyroid peroxidase gene expression (Tominaga *et al.*, 1991). In addition to this, thyrocytes produce IL-8 which can act as a chemoattractant for lymphocytes (Weetman *et al.*, 1992), IL-1 is able to increase production of IL-8 whilst IFN-γ reduced basal and IL-1 stimulated IL-8 production.

Therefore the thyroid cell has the potential to be an antigen presenting cell in that it is able to express class II antigens in particular in response to cytokines released by T-cells such as IFN-γ and TNF-α. In addition to this other regulatory cytokines are produced by and affect the function of thyroid epithelial cells and potentially infiltrating T-cells. These are T-cell products which along with raised TSH levels are general accompaniments to thyroid disease following destructive attack of the thyroid and the generation of hypothyroidism; therefore T-cells are very clearly involved in the initiation and perpetuation of experimentally induced autoimmune disease and human autoimmune thyroid diseases

These conditions are relevant to an on-going disease state but do not necessarily relate to a role in the initiation of the immune response. Initiation of the immune response may be due to a local thyroidal infection and subsequent tissue damage or due to a cross reactivity with environmental antigens, which may be perpetuated by the expression of class II antigens and costimulatory molecules on the thyroid cell. It cannot be assumed that conditions which enhance class II expression and propagation of an immune response are those which are responsible for the initiation of an immune response.

Although a number of antigens are implicated in human autoimmune thyroid diseases, in experimentally induced thyroid autoimmunity, thyroglobulin is the most commonly used autoantigen for induction of the disease in genetically susceptible mice although TPO can also be used but the susceptible strains are different (Kotani *et al.*, 1990). Given this specific requirement for particular MHC molecules, we have investigated the specificity of the antigen response for the H-2^k haplotype and been able to define a particular region that is recognised by T-cell clones and hybridomas derived from susceptible H-2^k mice. We have been able to define the residues involved in peptide binding to this class II molecule and the TCR, this enables us to speculate on the possible role of environmental antigens in the induction of thyroid and other autoimmune diseases. Further to this we have investigated the potential of thyroid epithelial cells as antigen presenting cells and attempted to delineate any potential differences in the presentational capacity of thyrocytes derived from H-2^k, high responder mice; which differ in their susceptibility to induction of experimental autoimmune thyroiditis.

2.0 Materials & Methods.

2.1 Materials.

Animals.

A) Mice.

BALB/c	NIMR, London, UK.
CBA/J	NIMR, London, UK.
CBA/Ca	NIMR, London, UK.
DBA/1	Harlan Olac, Bicester, Oxon, UK.
Parkes	UCMSM, London, UK.
SJL	Harlan Olac, Bicester, Oxon, UK.

All mice were supplied between the ages of 4-8 wks unless specified.

b) Rats.

Lou/C	Harlan Olac, Bicester, Oxon, UK.
Da	Harlan Olac, Bicester, Oxon, UK.
Wistar	UCMSM, London, UK.

Rats were supplied from the age of 10 wks.

Cell lines.

CH9	UCMSM, London, UK.
ADA2	UCMSM, London, UK.
YTS.177.9.6.1	Prof. H. Waldmann, Cambridge, UK.
CTLL	UCMSM, London, UK.

Tissue culture reagents.

RPMI	Gibco, Paisley, Scotland.
HBSS	Gibco, Paisley, Scotland.
Pen. / Strep.	Gibco, Paisley, Scotland.
L-Glutamine	Gibco, Paisley, Scotland.
NEAA	Gibco, Paisley, Scotland.
Na pyruvate	Gibco, Paisley, Scotland.
2-Me	Gibco, Paisley, Scotland.
Hepes	Gibco, Paisley, Scotland.
FCS	Gibco, Paisley, Scotland.

Cytokines.

r-mu-IFN-γ	Genentech, San Francisco, USA.
r-mu-TNF-α	Genentech, San Francisco, USA

Monoclonal antibodies and conjugates

Rab α-hu-T4	Dakopatts, Glostrup, Denmark.
Rat α-mu-I-A	Serotec, UK.
Rab F(ab')$_2$ α-rat IgG-FITC	Serotec,UK.

Buffers.

Phosphate buffered saline-10x - The following were dissolved in 1L of distilled water: 80g sodium chloride (NaCl), 11.5g di-sodium hydrogen orthophosphate (Na_2HPO_4), 2g potassium chloride (KCl) and 2g potassium di-hydrogen phosphate (KH_2PO_4). For a working dilution, dilute 100ml of 10x concentrate to 1L

Red cell lysing buffer - The following were dissolved in 1L of distilled water: 8.29g ammonium chloride (NH_4Cl) and 1g potassium hydrogen carbonate ($KHCO_3$). To lyse red blood cells in a spleen cell suspension, dilute cells in lysing buffer to approximately 5ml/ spleen and allow to stand at room temperature (RT) for 5 min before centrifuging at 200g for 10 min.

Paraformaldehyde buffer - Paraformaldehyde was diluted in PBS to a working concentration of 1% for fixation.

Chemicals and reagents.

3-Amino-1-2-4-triazole	Aldrich, Gillingham, Dorset, UK.
Bovine TSH (NISBC standard)	Dr. S. Bidey, Manchester, UK.
Concanavilin A	Sigma, Poole, Dorset, UK.
Complete Freund's adjuvant (H37 Ra)	Difco, East Molesey, Surrey, UK.
DNase-I	Sigma, Poole, Dorset, UK.
Formal saline	Merck Ltd, Butterworth, Leics,UK.
Insulin (human)	Pharmacy, Middlesex Hospital, London, UK.
Hydrocortisone	Sigma, Poole, Dorset, UK.
Transferrin	Sigma, Poole, Dorset, UK.
Pristane	Sigma, Poole, Dorset, UK.
Trypsin	Sigma, Poole, Dorset, UK.
L-Thyroxine	Sigma, Poole, Dorset, UK.

Radio labelled compounds.

(^{3}H) - Thymidine (5mCi)	ICN Flow, High Wycombe, Bucks, UK.
(^{125}I) - Deoxyuridine (500μCi)	Amersham, Bucks, UK.

Consumables.

Tissue culture plastics	Nunc, Life Technologies Ltd, Paisley, Scotland.

2.2 Methods.

2.2.1 Clones.

ADA2 - Clonotypically distinct from CH9, murine T-cell hybridoma reactive to murine Tg and cross reactive with human Tg (Rayner *et al.*, 1987). The clone is class II restricted and has a Th_1 phenotype secreting IL-2 and IFN-γ and is maintained in RPMI-5% at a cell density of $1x10^5$ - $1x10^6$ cells/ml.

CH9 - Murine T-cell hybridoma reactive to murine Tg and cross reactive with human Tg (Rayner *et al.*, 1987). The clone is class II restricted and has a Th_1 phenotype secreting IL-2 and IFNγ and is maintained in RPMI-5% at a cell density of $1x10^5$ - $1x10^6$ cells /ml.

CTLL - Murine T-cell clone, responsive to IL-2 and IL-4 (Gillis *et al.*, 1978 and Mossmann *et al.*,. 1986). It is maintained in RPMI-5% with rat spleen Con A supernatant (3%) at a cell density of $1x10^4$ - $1x10^5$ cells /ml.

YTS 177.9.6.1 - Rat B-cell hybridoma producing a non-depleting rat (IgG2a) anti-mouse CD4 McAb. Cells are maintained in RPMI-5% at a cell density of $1x10^5$ - $1x10^6$ cells / ml.

2.2.2 Animals.

Mice - These were supplied as specified previously and were held within the departmental animal facility for one week prior to use.

Rats - Outbred Wistar rats of both sexes were bred within UCMSM and used from an age of 3 months for the production of rat ConA (concanavilin A) supernatant for the maintenance of the CTLL cell line.

Female Lou/C rats were crossed with male Da rats to provide Lou x Da F1 hybrids for the production of monoclonal antibodies in ascites fluids. The rats were bred within UCMSM and used from an age of 10 wks.

2.2.3 Tissue culture.

All clones were routinely maintained in RPMI 1640 tissue culture medium with supplements and serum as shown on the next page.

RPMI-5%.(final working concentration in brackets)

-RPMI 1640
-1% L-Glutamine 200 mM (2mM)
-1% MEM (non essential amino acids) 100 x (1x)
-1% Sodium pyruvate 100 mM (1mM)
-0.5% Antibiotics (Penicillin / Streptomycin) 10000 IU/ml (50 IU/ml)
-0.1% 2-Mercaptoethanol 50 mM (50 μM)
-5% Heat inactivated foetal calf serum (FCS)

RPMI with the above supplementation and 5% FCS will be referred to as RPMI-5%, RPMI-10% represents the above supplementation with 10% FCS.

TEC medium 5% (final working concentration in brackets).

-1% MEM (non essential amino acids) 100x (1x),
-1% L-Glutamine 200 mM (2mM),
-0.5% Antibiotics (Penicillin / Streptomycin) 10000 IU/ml (50 IU/ml),
-5% Heat inactivated FCS.

TEC medium hormonally supplemented (in addition to the above medium).

-0.1% 2-Mercaptoethanol 50 mM (50 μM),
-1% NaI 100 μM (1μM),
-1% HEPES 100x (25mM),
-2% Insulin 100 IU/ml (20 IU/ml),
-0.1% Hydrocortisone 1000x 10 μM (10nM),
-0.1% Transferrin 1000x 5mg/ml (5μg/ml).

2.2.4 Antigens.

A) Preparation of mouse thyroglobulin (MTg).

Thyroid lobes from outbred Parkes mice were removed (2.2.7 G) and stored in PBS at -20°C until a large enough number were available to prepare thyroglobulin. Approximately 100 glands were required for each preparation.

Extraction - Thyroids were homogenised in PBS in a volume no greater than 10 ml, until the suspension was milky and all thyroid lobes had been disrupted. The extract was spun in a microfuge at 13000 rpm for 5 min at 4°C. The supernatant was then filtered through PBS wetted absorbent cotton wool which was then washed with a small amount of additional PBS and the final volume adjusted to a whole figure.

Precipitation - The supernatant was then transferred to a small beaker on a magnetic stirrer, stirring fast but ensuring not to cause foaming. Whilst stirring, saturated ammonium sulphate (SAS) was added dropwise to give a final concentration of 37% .The suspension was covered and allowed to stand at RT for 30 min before centrifuging and the precipitate

(mainly IgG), discarded. The supernatant was then brought to a final concentration of 45% SAS whilst stirring and allowed to stand covered for a further 1h at RT. The precipitate was then centrifuged as previously and the supernatant discarded, the precipitate containing the thyroglobulin was then washed in 45% SAS. Finally the precipitate was redissolved in PBS and dialysed against 5L of PBS at 4°C for 48 h with 3 changes of PBS. The amount of protein was calculated by measuring the absorbance at 280nm against a PBS blank using an LKB Ultraspec 4050, Pharmacia. An absorbance of 1 at 280nm of a 1cm cell = the concentration of protein in mg/ml. The thyroglobulin was adjusted to a concentration of 1mg/ml in PBS and filter sterilized using a 0.22μm filter and stored at -20°C until required.

B) Preparation of non-iodinated thyroglobulin (ATA-Tg)

The Parkes mice from which 3-amino-1-2-4-triazole (ATA)-Tg was to be prepared were pretreated for at least 6 wks with 1% ATA in their drinking water to deplete their thyroids of organic iodine. The method for preparation was exactly the same as for MTg.

C) Peptide synthesis.

Peptides were provided courtesy of Dr Mario Geysen, Chiron Mimotopes Pty. Ltd., Victoria, Australia.

Peptides were synthesized using N-α–Fmoc protected amino acids, with side chain protection if required. All peptide couplings were performed in polypropylene microtitre plates on a peptide synthesizer (9050; Milligen/Biosearch, Burlington,MA.) at 25°C over 16 h. F-moc deprotection of peptides followed by coupling was carried out until the target peptides were assembled. Following peptide assembly side chains were de-protected and the peptides cleaved into 0.1M phosphate buffer pH 7.0 at RT (Maeji *et al.*, 1990). Sets of overlapping peptides were obtained in 96 well plates in 150μl volumes for dilution. T4 (2553), $T4^-$ ve and benzyloxyl peptides were obtained in lyophilised form for dilution.

On arrival concentrated peptides were stored at -70°C until required for dilution, peptides were then diluted in PBS to the required concentration and aliquoted and stored at -20°C until used in assays.

2.2.5 Preparation of antibodies.

A) Production of monoclonal antibodies in rat ascites.

Lou x Da rats were primed one week before injection of cells with 2ml of pristane (2,6,10,14-Tetramethyl pentadecane) by intraperitoneal (i.p.) injection. Prior to injection cells for monoclonal antibody production, cells were grown *in vitro* and taken at late log phase growth. Cells were washed and resuspended in RPMI basal medium at a cell concentration of at least $4x10^7$ cells /ml such that greater than or equal to $4x10^7$ cells were injected in 1-2 ml of suspension *i.p.*

Ascites were harvested from one week after injection of cells Rats in which fluid was accumulating were generally over sensitive to external stimulation, often appearing gaunt in the face, with fur slightly out of condition. On standing on the haunches, fluid deposition was visible around the midrift and in the latter stages the rats became sensitive to handling. It was ensured that the rats were killed and the fluid taken aseptically before ascites accumulation became too uncomfortable. Ascites were either stored frozen at -20°C or prepared at once.

B) Preparation of monoclonal antibody from ascites.

A measured volume of ascites was placed in a beaker and left stirring whilst saturated ammonium sulphate (SAS) was added dropwise to make a 40% solution. The solution was then left standing at RT for 30 min before being aliquoted into eppendorf tubes and centrifuged using a microfuge at 13000 rpm for 5 min at 4°C. The supernatant was then discarded and the pellet washed in 40% SAS (in distilled water) as described above. Finally, the pellet was then dissolved in PBS and dialysed against 5L of PBS at 4°C for 48h (3 changes of PBS). The amount of antibody (IgG) was then calculated by measuring the absorbence at 280nm against a PBS blank using an LKB Ultraspec 4050, Pharmacia. An absorbence value of 1.0 at 280nm of a 1cm cell = 0.7 mg/ml Ig (assuming that all the protein present is immunoglobulin.

2.2.6 Cytokine assay.

A) CTLL proliferation assay for IL-2.

The CTLL line responds to IL-2 produced as result of T-cell activation by specific antigen. For hybridoma lines, proliferation is an unsuitable method for assessment of activation as the lines are continuously dividing. Plates containing 100µl supernatants were thawed at RT. CTLL cells in mid-log phase growth were washed twice to remove all traces of IL-2 used in maintenance of growth. Cells were then resuspended at a cell concentration of $1x10^5$ cells /ml and 100µl of suspension added to each well to be tested. A dose response curve of CTLL cells to IL-2 , including a negative control (medium only), was also set up to accompany the assay to ensure the cells were functioning correctly. The cells were then incubated for 24 h at 37°C, 5% CO_2 to ensure that the cells in the negative control had died off. The wells were then pulsed for 18 h with 1µCi (^{3}H)-thymidine, the cells harvested on to Titertek fibre filters using a Titertek cell harvester (Flow Labs.) and the degree of thymidine incorporation measured by placing individual discs in vials containing 2mls of scintillation counted using a Unit Technologies Packard, Minaxi Tricarb 4000 series beta emission counter.

2.2.7 *In vitro* protocols.

A) Production of rat spleen Con A supernatant for maintenance of CTLL line.

Outbred Wistar rat spleens were removed aseptically into RPMI-5% in a petri dish. Under sterile laminar flow conditions the spleens were disaggregated using blunt forceps to make a single cell suspension washed twice in fresh medium and the cells counted. The cells were then resuspended in fresh RPMI basal medium with glutamine, antibiotics and ConA (2.5μg/ml) at a cell concentration of $5x10^6$ cells/ml. The suspension was then placed in $80cm^2$ tissue culture flasks and incubated flat at 37°C, 5% CO_2 for 30h. After 30h the ConA activity was blocked using 2-methyl-D-mannopyranoside (20 mg/ml). Following this the cells were removed by spinning at 3-5000 rpm and the supernatant collected, aliquoted and stored unsterilised at -20°C until required. When required the supernatant was thawed and filter sterilised using Minisart plus 0.22μm filter. New batches of rat IL-2 supernatant were titrated against CTLL cells to determine the optimal percentage for growth prior to use.

B) Preparation of peritoneal feeder cells for establishment of CTLL line.

BALB/c mice ($H\text{-}2^d$) spleen cells were used to act as accessory cells in the establishment of the CTLL line. The function of the accessory cell is to provide growth factors and to maintain cell density. Donor mice were killed by cervical dislocation placed in a flow hood and the abdominal skin sprayed with 70% ethanol to maintain aseptic conditions. The abdominal skin was cut and paired away to expose the peritoneum. The peritoneum was lifted using forceps and injected with 10ml of cold RPMI basal medium. Once the needle was removed the abdomen was agitated to suspend the peritoneal cells and the fluid slowly withdrawn again using the needle and syringe. The cells were washed twice by centrifuging at 200g for 10 min, counted and resuspended to $4x10^6$ cells/ml. To this, freshly thawed CTLL cells were added at a concentration of $2x10^4$ cells/ml, giving a final concentration of $2x10^6$ feeders : $1x10^4$ CTLL cells. Volumes (1ml) were placed in 24 well tissue culture plates and the CTLL cells allowed to grow and the feeder cells to die out.

C) Preparation of accessory cells for antigen presentation to T-cells.

The appropriate number of syngeneic mice were killed by cervical dislocation and the spleens aseptically removed and placed in RPMI-5%. The spleens were then teased apart using sterilized, blunt forceps to release all the cells (approx $2x10^7$/spleen) into the medium. The resultant supernatant was then washed by centrifuging at 200g for 10 min. The red blood cells present were lysed by resuspending the cells in red blood cell lysing buffer (see section 2.1) and washed twice. The cells were then resuspended to a small volume and counted, and then irradiated by a 2000 Rad exposure using a ^{60}Co source of gamma radiation to prevent further replication. Cells were then diluted to the required concentration and stored on ice until required.

D) Preparation of primed lymph node cells

After the appropriate immunization protocol (2.2.8 B&C), mice were killed by cervical dislocation and the popliteal lymph nodes removed under aseptic conditions into RPMI-5% on ice. The lymph nodes were then transferred to fresh culture medium and the nodes disrupted using sterile blunt forceps. The resultant suspension was then washed twice at 200g for 10 min and the cells counted. The cells were then resuspended to the appropriate concentration for use in a lymph node T-cell proliferation assay (2.2.7 E).

E) Lymph node T-cell proliferation assay.

Using 96 well flat bottomed microtitre plates, 100µl of primed lymph node cell suspension (responder cells), containing $2x10^5$ cells, was added to each well. The final volume of 200µl was made up by the addition of 100µl of the appropriate concentration of antigen/s or medium to give the correct concentration/ml. Positive and negative controls were also be included on the same plate. The cells were then incubated at 37°C for 72 h in humidified, 5% CO_2 atmosphere and then pulsed for 18 h with 1µCi (^{3}H)-thymidine. The wells harvested and the degree of thymidine incorporation measured as described previously.

F) T-cell hybridoma IL-2 release assay.

The responder cells are T-cell hybridomas taken in mid-log phase growth which have been washed and resuspended to give a final cell concentration of $2x10^5$ cells per 50 µl. To this 100µl of irradiated syngeneic spleen cell suspension containing $2x10^6$ cells was added to each well. The final volume was made up by the addition of 50µl of antigen to give the correct concentration/ml in 200µl volume. Following a 24h incubation period in the presence of splenic antigen presenting cells and specific antigen, 100µl supernatants were taken using a multichannel pipette and placed in the corresponding position on a fresh 96 well plate. Supernatants were then frozen at -70°C. The freezing step is essential to kill any of the hybridoma cells that may have been carried over since the cells would grow in the read-out assay and thus lead to false positive results. Plates can be stored at -20°C until required for reading (2.2.6 A)

G) Thyroid epithelial cell culture.

Either CBA/Ca or CBA/J mice were asphyxiated by lethal CO_2 administration and removed to aseptic conditions. The mice were swabbed with 70% ethanol to dampen the fur and pinned out by the snout and forearms so that the thorax was elevated and taut . The skin was removed to expose the salivary glands which were then removed with the layer of muscle which covers the gland, therefore exposing the thyroidal lobes on the trachea. Below the gland the trachea was lifted, cut and the muscles pared off such that the whole trachea could be lifted away and cut above and below the gland so that it was removed whilst still on the trachea. This was then placed in a petri dish on ice containing TEC medium 5%. Once all glands had been removed the dish was placed in a laminar flow hood and the thyroid lobes were removed from the trachea using curved forceps and placed in a drop of

TEC medium 5% in a fresh sterile petri dish. The lobes were then roughly dissected using small surgical scissors to assist digestion and were then placed (approx 1ml per mouse) in a freshly made solution of 1.5 mg/ml (0.32 U/ml) collagenase in a sterilized glass 30 ml universal containing a magnetic stirrer bar. This was then placed in a 37°C water bath on a heated magnetic stirrer, the speed was such that a small vortex was created, for approximately 30 min.

The digestion was checked at 20 and 25 min to ensure the thyroids had not over digested. The optimal digestion was such that the lobes had nearly completely dissociated into follicles but not into a single cell suspension. Lobes that had not completely digested could usually be disrupted by pipetting. The collagenase digestion was then filtered using sterilized 100μm mesh placed over a small beaker, and the resultant suspension transferred to a universal and diluted using TEC medium 5% to inactivate the enzyme and prevent further digestion. The suspension was centrifuged at 200g for 10 min to pellet the follicles and then resuspended to an appropriate volume for counting. The number of follicles was assessed by removing a 100μl aliquot of suspension and placing it in a well of a 96 well plate. Once the follicles had settled they were counted and the total number of follicles assessed. This suspension was then diluted to give a final follicle concentration of 50 follicles per well in a 100 μl volume and this suspension was then plated out onto 96 well plates.

Follicles were incubated for 24 h before the removal of the culture medium and replacement with medium +/- r-murine-IFN-γ (100 U/ml unless otherwise indicated) and/or 1 μM sodium iodide (NaI). Other additions used at this time point which are outlined in the results text are 1% ATA (prepared as a 100x stock in RPMI) and 0.1 μg/ml TSH (prepared as 500x stock in HBSS). Cells were then incubated for a further 72 h before the addition of medium +/- r-murine TNF-α and/or exogenous antigen. Thus thyroid epithelial cell cultures were assessed at 5 days culture, at this point viable follicles had adhered and spread over the culture surface and the level of fibroblast contamination was minimal. For antigen presentation assays TEC's were used at day five of culture and syngeneic spleen cells were prepared (2.2.7 C) on the day for use as presentation controls.

H) Thyroid epithelial cell presentation assay.

TEC's were prepared as outlined in (2.2.7 G). On day five, thyroid epithelial cells were used as antigen presenting cells following the addition of exogenous antigen 18 h prior to assay to allow processing and presentation by the cells. On day five, syngeneic spleen cell control cultures were also prepared and pulsed with the appropriate antigens prior to the addition of $2x10^5$ Tg reactive hybridoma cells, CH9 or LNC. Cultures were incubated for 18h and the supernatants removed to corresponding wells on separate 96 well plates before freezing prior to assay for IL-2 activity by CTLL cells as outlined in 2.2.6 A.

I) Thyroid epithelial cells for flourescent staining.

Cells were prepared exactly as stated in 2.2.7 G, except that cultures were plated into 24 well culture dishes at an approximate follicle concentration of 200 follicles per well in 1ml of TEC medium 5%. At the day five time point following incubation with cytokines but not specific antigen, the culture medium was removed and the cells washed with basal RPMI before the medium was replaced by Ca^{2+}/Mg^{2+} free HBSS plus the relevant cytokines being used in the culture medium. The cultures were then left overnight (18h) in a 37°C incubator after which time most of the cells had rounded and detached from the culture plates. The cells were then removed from the plates and any remaining were removed by using a 0.04% trypsin/HBSS (v/v) solution. All cells were then centrifuged at 200g for 10 min and then resuspended in Ca^{2+}/Mg^{2+} free HBSS containing 10µg/ml DNase-1 for staining. The presence of DNase prevented the follicular cells from sticking together whilst staining. Cells were stained for the presence of surface class II using 1:50 rat anti-mouse Ia^k affinity purified antibody first layer on ice (40 min) followed by 1:100 FITC conjugated rabbit anti-rat Ig $(Fab')_2$ fragment, in the dark and on ice (40 min). Background staining by the $(Fab')_2$ fragment were assessed by staining a separate population of cells in the absence of a first layer antibody. Finally cells were fixed using 1% paraformaldehyde before assessment on an Axiophot fluorescence microscope (Zeiss).

2.2.8 *In vivo* protocols.

A) Preparation of antigen/CFA immunising agent.

Either MTg (1mg/ml in PBS) or peptide (200µg/ml in PBS) was mixed 1:1 with complete Freund's adjuvant (CFA). Antigen was added dropwise, thoroughly mixing between each step, until all antigen had been added and the mixture began to thicken and look milky white. The mixture was then vibrimixed for 10 min and placed on ice for 20 min and then vibrimixed for a further 10 min before testing. To test whether an emulsion had formed a small aliquot was dropped onto distilled water in a petri dish. If a true emulsion had formed, the drop remained entirely intact without any oiling of the water surface. The concentration of MTg was such that in a 100µl injection, either into the base of the tail or in two footpads, the amount of antigen delivered was 50µg. For peptide the amount of antigen delivered in a 100µl dose was 10µg.

B) Priming of day-8 lymph node cells.

On day 0, mice were immunised under anaesthetic in the hind footpads,with 50 µl per footpad of antigen/CFA emulsion delivering a total dose of 50µg of MTg or 10µg of peptide. The mice were maintained in the animal facility until day eight when they were killed by cervical dislocation and the popliteal lymph nodes removed under aseptic condition into RPMI-5% on ice. These were then processed as described in 2.2.7 D.

C) Priming of day-16 lymph node cells.

On day 0, mice were immunised under anaesthetic subcutaneously in the base of the tail, with 100 µl MTg/CFA emulsion delivering a dose of 50µg MTg or 10 µg peptide. On day seven the mice were immunised in the hind footpads with 50µl per footpad of antigen/ CFA emulsion delivering a total dose of 50µg of MTg or 10µg of peptide. The mice were maintained in the animal facility until day sixteen when they were killed by cervical dislocation and the popliteal lymph nodes removed under aseptic condition into RPMI-5% on ice. These were then processed as described in 2.2.7 D.

D) Tolerisation with non-depleting anti-CD4.

In studies where anti-CD4 was used to abrogate responses, 2mg (0.2ml) of antibody was given i.v. on the first day, before immunisation with antigen and the i.p for two more consecutive days. Following this 2mg of anti-CD4 was given i.p on alternate days for another three or six doses depending on whether the lymph nodes were day eight or day sixteen primed, respectively.

Results.

3.0 *In vitro* analysis of a T-cell epitope recognised by Tg reactive T-cell hybridomas.

3.1 Localisation of the T-cell epitope to a particular thyroxine containing region.

Previous work by Champion *et al.*, (1987) has demonstrated that in the H-2A^k restricted CBA/J mouse the iodination levels of Tg are critical to induction of EAT. This implies that T-cell residues involved in the induction of EAT may be iodinated or be near iodination sites within the Tg molecule. The publication of the amino acid sequence for human Tg (Malthiery & Lissitzky 1987) allowed the synthesis of a panel of 12 mer overlapping peptides which spanned the four hormonogenic regions of Tg to test this hypothesis. The response of a T-cell hybridoma (CH9) derived from a pathogenic MTg/HTg reactive H-2A^k restricted T-cell clone (Rayner *et al.*, 1987) to these peptides was investigated. Figure 3.1 shows that CH9 recognises a distinct region of three 12 mer peptides covering a total of fourteen residues from leucine at position 2546 to alanine at position 2559 (LEHSTDDT4ASFSRA).

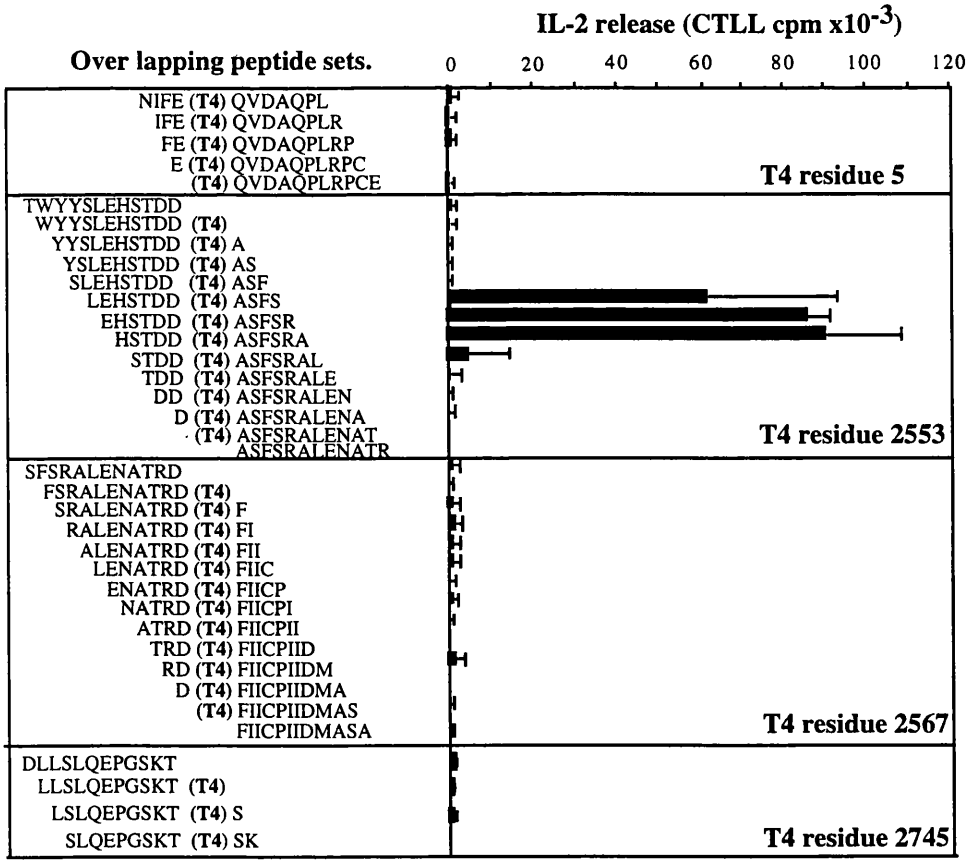


Figure:- 3.1. Localisation of the T-cell epitope to a particular thyroxine containing region.

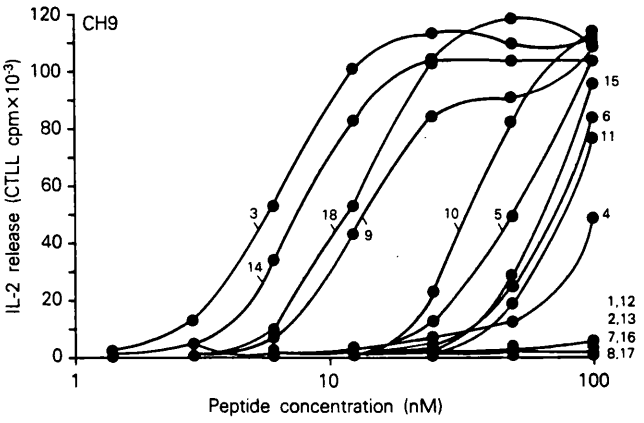
Response of 2x10^5 CH9 cells per well to sets of 12 mer overlapping peptides covering the four hormonogenic sites of Tg. The final peptide concentration 2.5µg/ml, supernatants taken after 24h, for assessment of IL-2 release. Results shown are average of triplicate cultures +/- SEM.

This same region is shown to be recognised by ADA2 a clonotypically distinct, Tg reactive T-cell hybridoma, although the pattern of recognition is slightly different to CH9 (Champion *et al.*, 1992).

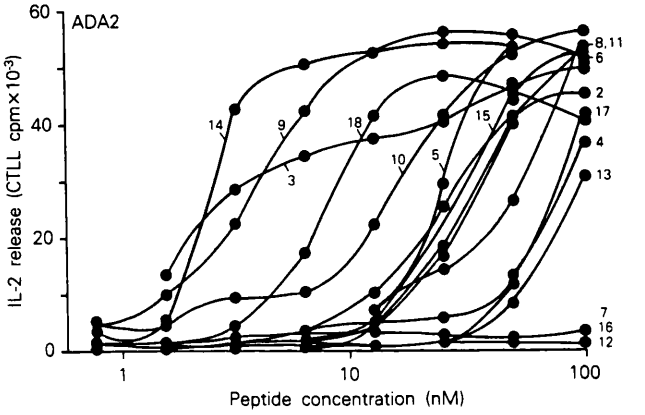
3.2 Definition of the minimal epitope.

This data permitted the subsequent definition of the minimal epitope recognised by CH9 and ADA2. The experiments shown in Fig.3.2 were conducted by and are reproduced here by kind permission of Dr B. Champion (Champion *et al.*, 1992) and have been included here for continuity. The data clearly show that the minimal epitope length that is recognised by both T-cell hybridomas is one of nine amino acids, the sequence being DD(T4)ASFSRA. Peptides of eight amino acids or less were all non-stimulatory over a range of 1-100 nM (data not shown). By titrating out the response to these peptides over the range 1-100 nM (Fig 3.2 ***a***& ***b***) the ED50 values were calculated (the concentration of peptide required to give a half maximal response) this enabled the determination of the peptides that gave the best responses (Fig 3.2 ***c***). Four peptide sequences were shown to give very good reproducible stimulation of the hybridomas, these were numbers 3). HSTDD(T4)ASFSRA, 9) STDD(T4)ASFSRA, 14) TDD(T4)ASFSRA and 18) DD(T4)ASFSRA. Peptides with an extension of the amino acids C-terminal to the minimal epitope served to reduce the peptide potency for both CH9 and ADA2. Loss of amino acids from the C-terminal of the minimal epitope drastically affected the peptide potency for CH9 but had a far less dramatic effect on the response of ADA2 to the peptide. This implies that the minimal epitope for ADA2 may be shifted by at least one or two residues towards the N-terminus. This may account for the apparent difference in recognition by CH9 and ADA2 of the 12 mer overlapping sequences shown in Champion *et al.*, 1992. The addition of residues N-terminal to the minimal epitope appear to have little effect on the overall peptide potency. Figure 3.2 is shown on the next page.

a)



b)



c)

Peptide	Sequence	ED_{50} (nM) CH9	ADA2
2	E H S T D D T4 A S F S R	>1250	30
7	E H S T D D T4 A S F S	>1250	>500
3	H S T D D T4 A S F S R A	6	2.5
8	H S T D D T4 A S F S R	>1250	48
12	H S T D D T4 A S F S	>1250	>500
4	S T D D T4 A S F S R A L	100	72
9	S T D D T4 A S F S R A	14	3.4
13	S T D D T4 A S F S R	>1250	85
16	S T D D T4 A S F S	>1250	>500
5	T D D T4 A S F S R A L E	52	23
10	T D D T4 A S F S R A L	34	14
14	T D D T4 A S F S R A	8	2.2
17	T D D T4 A S F S R	>1250	70
6	D D T4 A S F S R A L E N	70	32
11	D D T4 A S F S R A L E	75	32
15	D D T4 A S F S R A L	62	25
18	D D T4 A S F S R A	12	7.7
1	P L R Q G G G G G G G G	>1250	N.T.

Figure:- 3.2. Definition of the minimal epitope.

CH9 *(a)* and ADA2 *(b)* dose response curves to T4 containing peptides of different length representing the hormonogenic site at position 2553. The responses of CH9 and ADA2 to the T4 containing peptides are represented as the concentration required to stimulate a half-maximal response (ED_{50}) *(c)*. Peptides of 5,6,7 or 8 amino acids were all non-stimulatory (data not shown). Results shown are average of triplicate cultures. **(Results are reproduced with kind permission of Dr B. Champion, taken from Champion *et al* 1991).**

3.3 The essential role of thyroxine in epitope recognition.

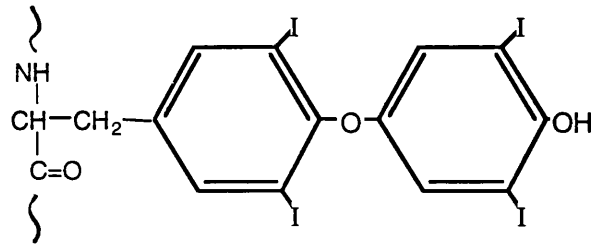
3.3A) Peptides lacking thyroxine at residue 2553 are non-stimulatory.

Given this information concerning Tg peptide recognition and previous knowledge about the requirement for fully iodinated Tg in the induction of EAT, it was necessary to ensure that the thyroxine (T4) residue contained within the peptide was necessary for T-cell recognition and activation. To determine whether the T4 residue was critical for stimulation of the hybridoma, CH9, three peptides were taken based on the 9 mer minimal epitope DD(T4) ASFSRA. These were the minimal epitope itself, a 10 mer and an 11 mer created by the addition of one and two amino acids to the C-terminus respectively. For each of these peptides the T4 residue was substituted with each of the naturally occurring amino acids to determine whether any could replace or partially replace the thyroxine residue function. It was found that no amino acid including tyrosine which is the acceptor residue in thyroxine formation, was able to replace or even partially restore the function of the T4 residue in any of the peptides (Fig 3.3A ***a, b*** & ***c***). Panel ***d***) show a dose response curve for the ADA2 hybridoma to an 11 mer $T4^{+}$(2553) peptide over the range 0.08 - 20 μg/ml. It can be clearly seen that ADA2 also requires the presence of the thyroxine residue (75% maximal response approx 0.8 μg/ml) as a comparable dose response curve for the $T4^{-}$(2553) peptide induces no response at any concentration tested.

Structure of $T4^{-}$(2553) peptide: STDD(Y)ASFSRA.

Structure of $T4^{+}$(2553) peptide: STDD(T4)ASFSRA, where the T4 side chain is:

$T4^{+}$ SIDE CHAIN



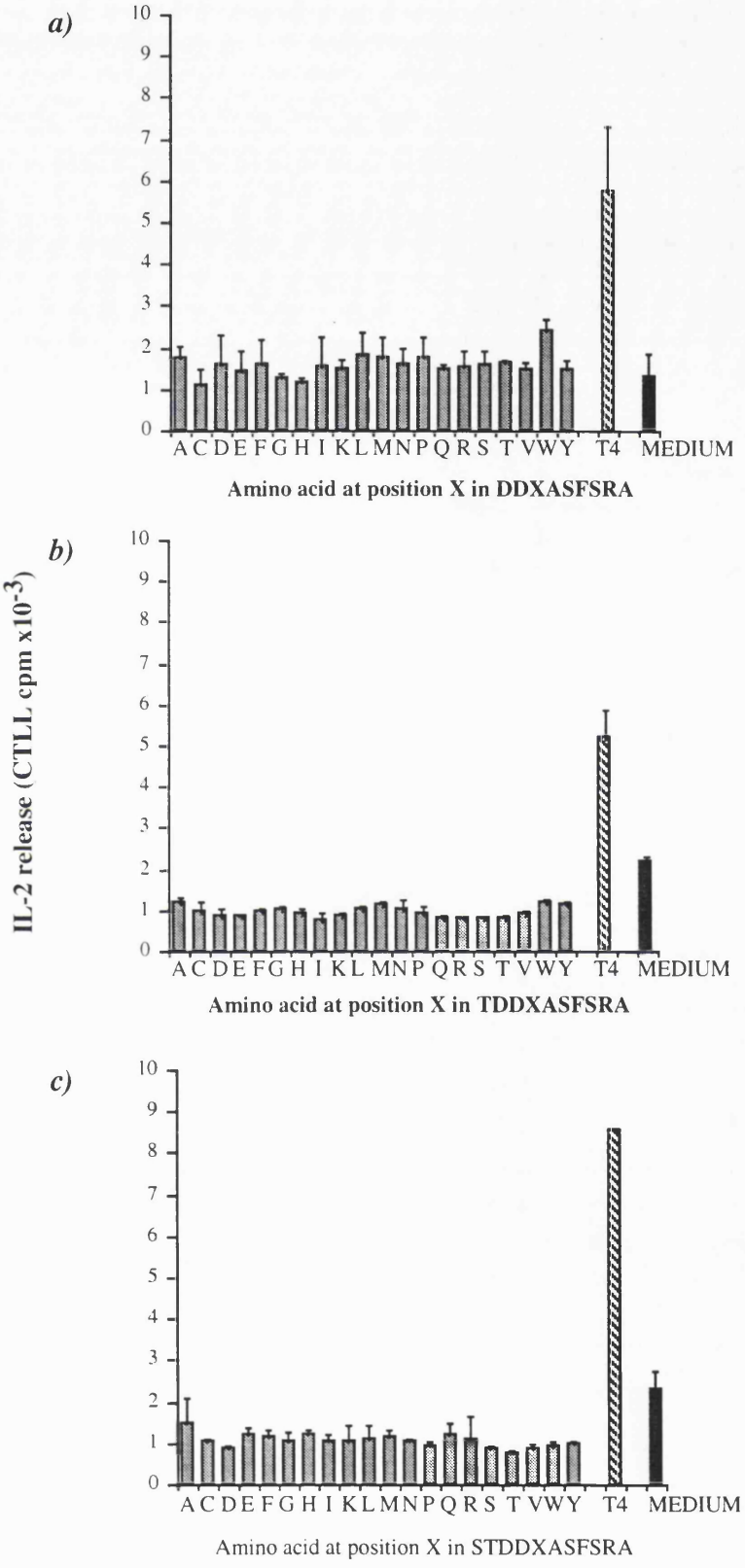


Figure:- 3.3A. Peptides lacking thyroxine at position 2553 are non-stimulatory.

Response of $2x10^5$ CH9 to either 9,10 or 11 mer peptides at a final concentration of 2.5 µg/ml. *(a)* 9 mer DD**X**ASFSRA, *(b)* 10 mer TDD**X**ASFSRA, *(c)* 11 mer STDD**X**ASFSRA where **X** is the amino acid indicated in the graph (-▨-). T4 substitution control shown (-▧-), medium control shown (-■-).

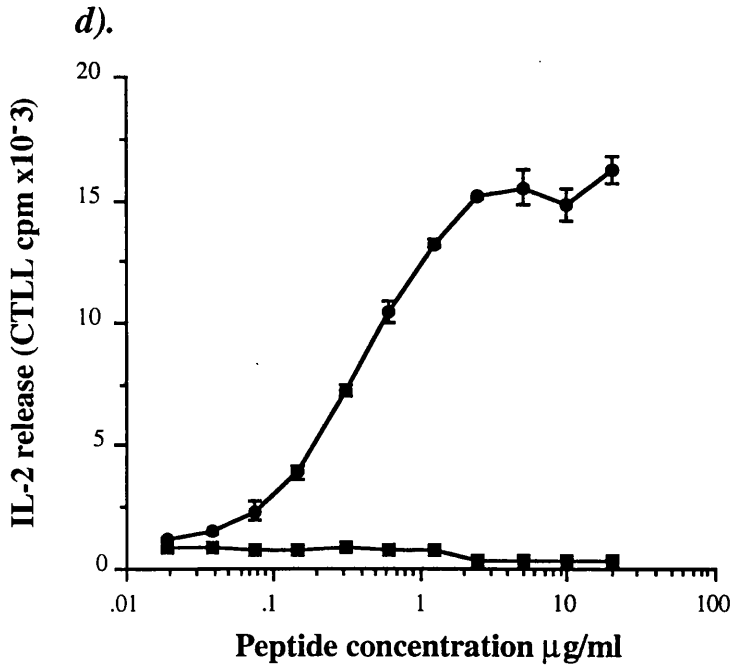


Figure:- 3.3A. Peptides lacking thyroxine at position 2553 are non-stimulatory.
(d) ADA2 response to 11 mer peptides T4^{+}(2553) (-●-) and T4^{-}(2553) (-■ -) over the range 0.08-20 µg/ml stimulatory peptide. Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM.

3.3B) De-iodinated thyroxine (benzyloxyl) containing peptide is non-stimulatory.
This role was investigated further by substituting the thyroxine side chain with a benzyloxyl group which leaves the backbone aromatic ring structure without the four iodination sites i.e. de-iodinated thyroxine (benzyloxyl peptide, Bz(2553)). The structure of this peptide is **STDD(Bz)ASFSRA** where the Bz side chain is:

BZ SIDE CHAIN

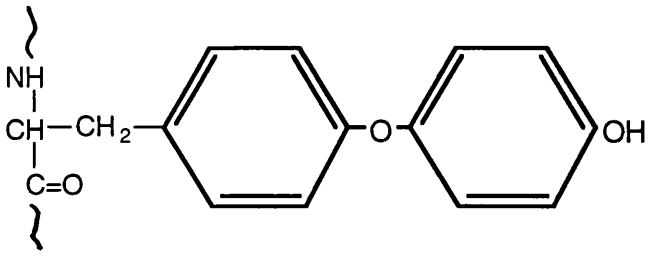


Figure 3.3B, panel *a)* shows that this peptide is totally non-stimulatory to CH9 at all concentrations tested 0.2-10 µg/ml. This dose response curve is shown alongside that for an un-substituted 11 mer peptide STDD(T4)ASFSRA which exhibits a 75% maximal response between 0.2-0.3 µg/ml. It will be recalled from 3.3A that even a concentration of 2.5µg/ml, peptides containing T4 substituted with any of the natural amino acids including the acceptor tyrosine were non-stimulatory. The response of ADA2 to the benzyloxyl peptide is shown in panel *b)* and like CH9 is not stimulated by this peptide over the range of concentrations tested 0.08 - 20 µg/ml.

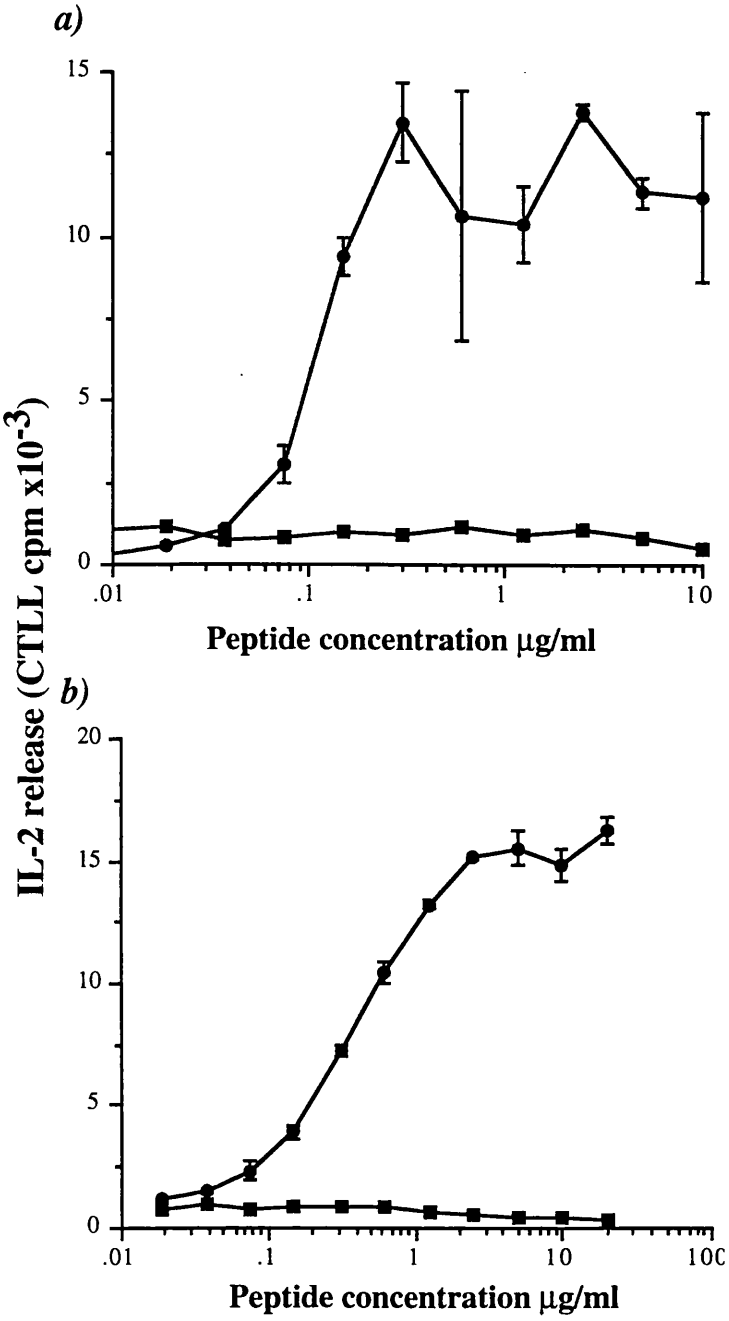


Figure:- 3.3B. De-iodinated thyroxine (benzyloxyl) containing peptide is non-stimulatory.

(a) Response of $2x10^5$ CH9 to $T4^+$(2553) (-●-) and Bz(2553) (-■-) peptides over the range 0.02 - 10 µg/ml final concentration. *(b)* Response of $2x10^5$ ADA2 to $T4^+$(2553) (-●-) and Bz(2553) (-■-) peptides over the range 0.08 - 20 µg/ml final concentration. Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM.

3.3C) T4$^-$(2553) and Bz(2553) peptides do not block the recognition of T4$^+$(2553) peptide.

In order to determine whether the T4$^-$(2553) or Bz(2553) inhibitory peptides could block the response of CH9 to iodinated stimulatory peptide T4$^+$(2553) a series of experiments were conducted, the results of which are shown in figure 3.3C ***a)*** & ***b)***.

In these experiments, the potentially inhibitory peptides were tested at concentrations up to 20μg/ml for their ability to block the response of CH9 to an inhibitable concentration (0.2μg/ml) of the stimulatory peptide. Potential inhibitor peptide concentrations greater than 20μg/ml were not tested although it may be reasoned that some degree of inhibition would be expected at a 100-fold excess of inhibitor concentration over stimulator peptide. A series of experiments were carried out the results of which are shown in figure 3.3C. Panel b) shows the effect of pre-incubation of syngeneic APC's with 20 μg/ml T4$^-$(2553) peptide for 1 h at 37°C. Some APC's were then washed to remove excess unbound peptide or left unwashed and the effect on their capacity to present T4$^+$(2553) peptide to CH9 assessed. The T4$^-$(2553) peptide was unable to block (washed) or competitively inhibit (not washed) the presentation of T4$^+$(2553) peptide to CH9 at the concentrations tested.

We also tried to inhibit the response of CH9 to T4$^+$(2553) peptide with Bz(2553) peptide a) Syngeneic splenic antigen presenting cells were pre-incubated as above, at two different concentrations of potential inhibitor peptide of 5 and 20μg/ml. Data is shown for pre-incubation with inhibitor peptide at 5μg/ml but the results are identical at 20μg/ml (data not shown). Again APC's were either washed or left unwashed to determine whether the peptide was able to block or competitively inhibit T4$^+$(2553) peptide binding to class II. At all concentrations tested the potential inhibitor peptides have no effect on stimulation by T4$^+$(2553).

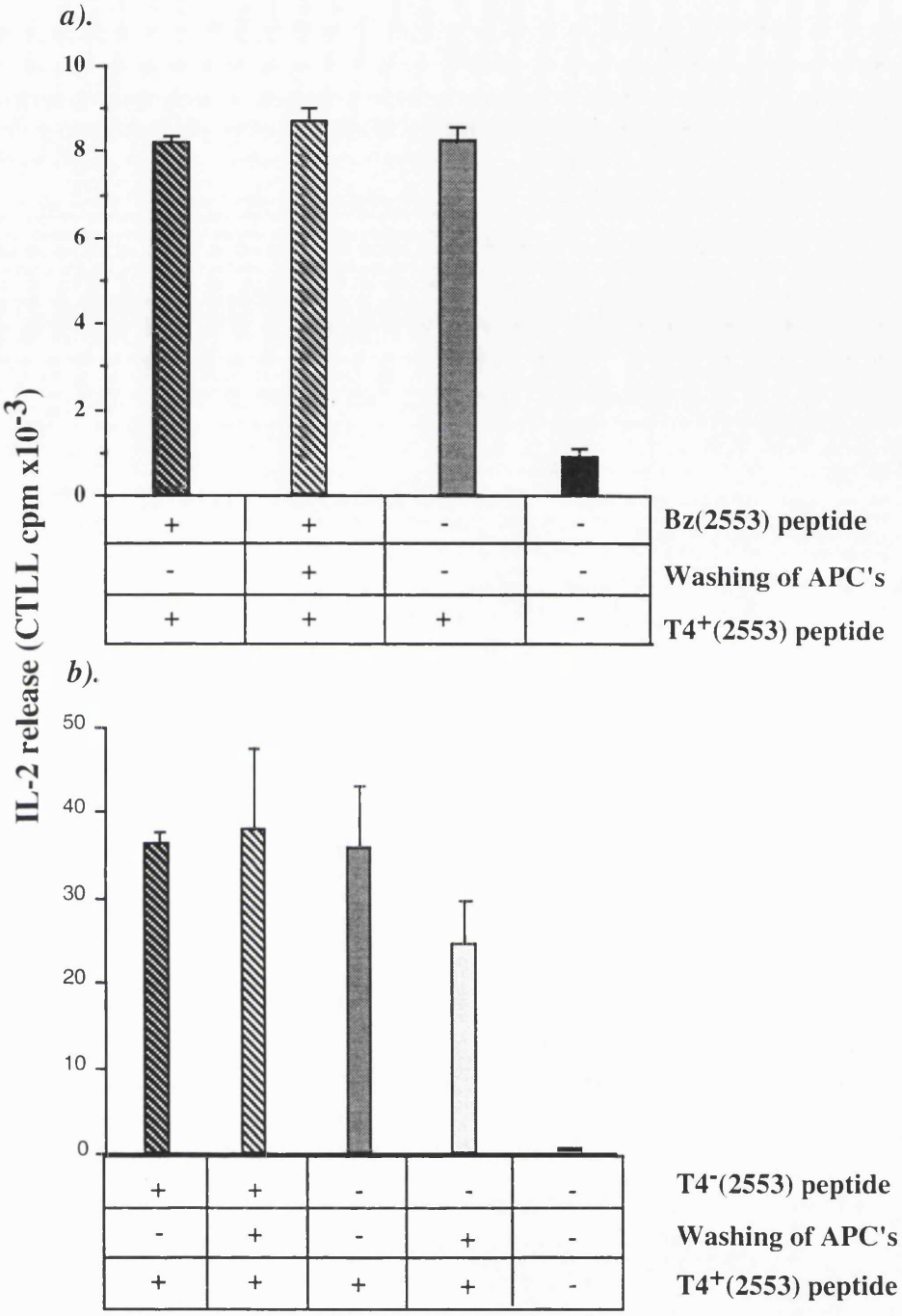


Figure:- 3.3C. T4⁻(2553) peptide and benzyloxyl peptides do not block recognition of T4⁺(2553) peptide.

(a) Response of $2x10^5$ CH9 to 0.2 µg/ml T4⁺(2553) peptide following 1 h pre-incubation of syngeneic APC's with 5 µg/ml Bz(2553) peptide. After incubation APC's were either not washed (-▨-) or washed (-▨-) to remove extraneous blocking peptide. T4⁺(2553) control shown (-▨-), medium control shown (-■-). *(b)* Response of $2x10^5$ CH9 response to 0.2 µg/ml T4⁺(2553) peptide following 1 h pre-incubation of syngeneic APC's with 20µg/ml T4⁻(2553) peptide Following incubation APC's were either not washed (-▨-) or washed (-▨-) to remove extraneous blocking peptide. T4⁺(2553) controls shown (-▨-) & (-□-), medium control shown (-■-). Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM.

3.3D) Anti-thyroxine antibody is able to block recognition of T4+(2553) peptide.

At this stage it was impossible to delineate whether the T4 residue is binding TCR or the MHC II. However it was possible to determine whether the T4 residue is exposed and thus potentially available for binding to TCR by looking at the inhibition of peptide stimulation of CH9 using polyclonal antibodies to human thyroxine. Syngeneic APC's were pre-incubated with a low but stimulatory concentration of T4+(2553) peptide (0.2 μg/ml) for one hour at 37°C. Immediately following this, varying doses of anti-thyroxine antibody were added and the cells incubated for one hour at 4°C. CH9 responder cells were then added and incubated at 37°C for 24 h before supernatants were removed for testing. Figure 3.3D clearly shows that this response is inhibitable by anti-thyroxine antibody in a dose-dependent manner. CH9 stimulated by a low concentration (0.2mg/ml) of T4+(2553) peptide is maximally inhibited by an antibody concentration of approximately 115 μg/ml (dilution of 1/105) and the concentration at which 50% inhibition occurs is 40 μg/ml (dilution of approximately 1/300).

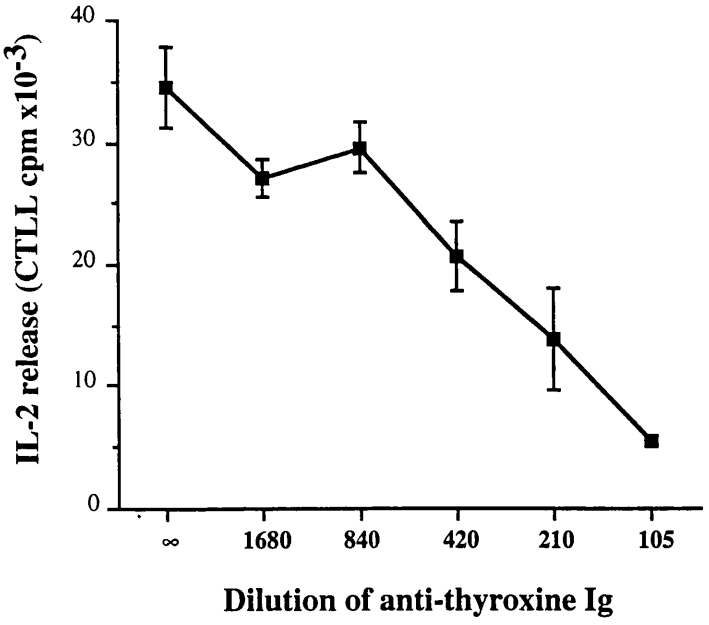


Figure:- 3.3D. Anti-thyroxine antibody is able to block recognition of T4+(2553) peptide.

Response of $2x10^5$ CH9 to T4+((2553) peptide at a limiting concentration (0.2 μg/ml) in the presence or absence of reciprocal dilutions of anti-thyroxine antibody (12.1 mg/ml neat). Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM.

3.3E) Responses blocked by anti-thyroxine can be reversed in the presence of free thyroxine.

In order to see whether the inhibitory effect of the polyclonal anti-T4 was related to its specificity for thyroxine. The above experiment was conducted in the presence of excess thyroxine. Free thyroxine at 1-5 μg/ml was added to the APC cultures following pre-incubation with peptide but before addition of anti-thyroxine antibody. Fig 3.3E) shows the same dose response to anti-thyroxine as shown in Fig.3.3D but in the presence of free T4 at concentrations greater than or equal to 1 μg/ml, the inhibition effect of the anti-T4 antibody was reversed. Inhibition was also seen to a lesser extent at a concentration of free T4 of 0.4 μg/ml (data not shown).

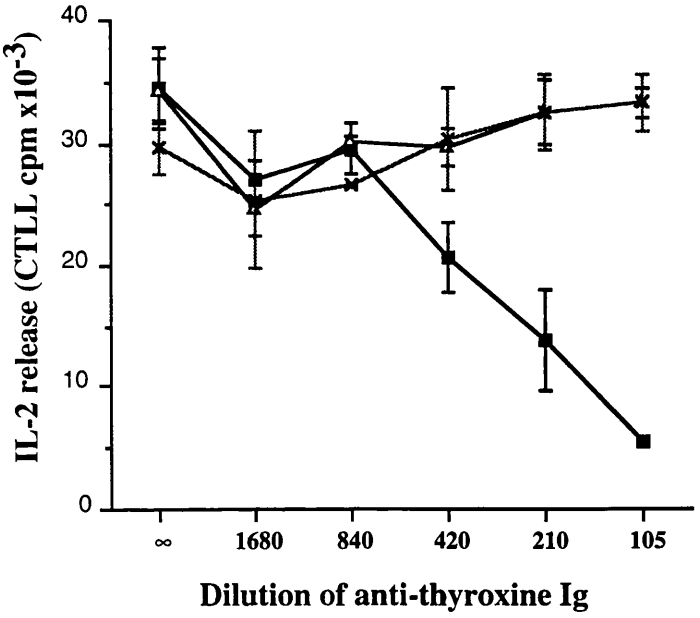


Figure:- 3.3E. Responses blocked by anti-thyroxine can be reversed in the presence of free thyroxine.

Response of $2x10^5$ CH9 to a limiting concentration (0.2μg/ml) of stimulating $T4^+$(2553) peptide in the presence or absence of dilutions of anti-thyroxine antibody (12.1 mg/ml neat). The effect of the addition of free T4 at 1 μg/ml (-Δ-), 5μg/ml (-**X**-) and no free T4 (-■-) are shown. Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM.

3.3F) Free thyroxine is non-stimulatory.

To exclude the possibility that free thyroxine acts as a hormone or perhaps a ligand for the TCR, might stimulate the T-cell hybridoma directly, CH9 was incubated directly with free T4 in the presence of APC's. Figure 3.3F clearly shows that free T4 over a range of concentrations from 0.75 - 25 µg/ml had no effect on the IL-2 release in the presence or absence of stimulatory $T4^+$(2553) peptide.

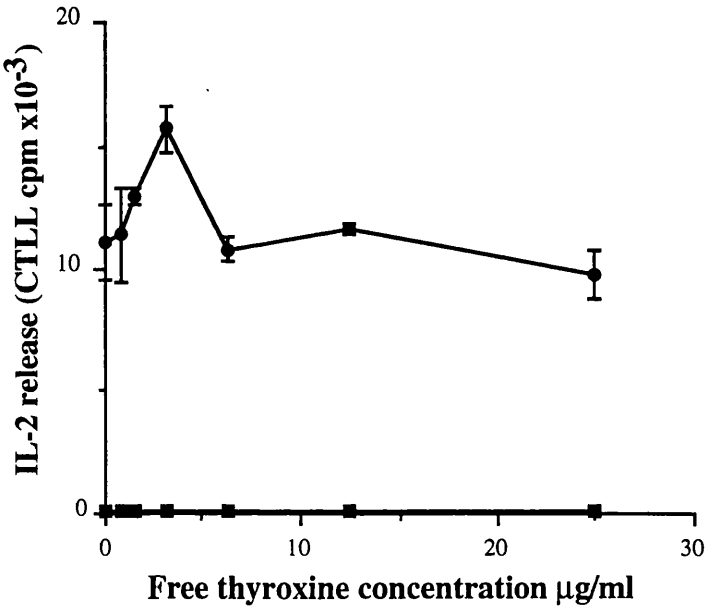


Figure:- 3.3F. Free thyroxine is non-stimulatory.

Response of $2x10^5$ CH9 to a dose response curve of free thyroxine in the presence (-●-) or absence of (-■-) 5µg/ml $T4^+$(2553) peptide. Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM.

3.4 Further analysis of T-cell interactions with T4+(2553) peptide.

3.4A) Anti-class II (H-2A^k) antibody is able to block recognition of the T4+(2553) peptide.

To confirm that the response is indeed class II restricted. It has previously been shown that the response of CH9 to the T4 containing peptide covering the T4 residue 2553 on the human thyroglobulin sequence was class II restricted, APC's were pre-pulsed with T4+(2553) at a concentration of 0.25 µg/ml and then added to hybridoma cells in the presence of the anti-class II monoclonal antibody, OX6, which is specific for monomorphic determinants on the rat class II molecule but cross reacts with murine H-2A$^{k\&s}$. IL-2 release by CH9 was completely inhibited by the anti-class II antibody.

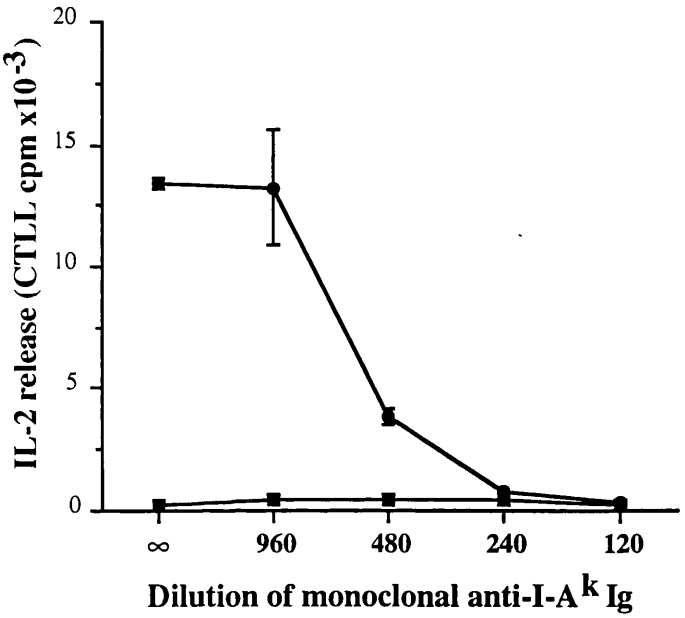


Figure:- 3.4A. Anti-class II (H-2Ak) antibody is able to block recognition of T4+(2553) peptide.

Response of 2x10^5 CH9 in the presence (-●-) or absence (-■-) of T4+(2553) peptide at a limiting concentration (0.2 µg/ml), showing the effect of dilutions of anti-class II (H-2A^k) antibody. Supernatants taken after 24h., for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM.

3.4B) A Vβ8 TCR is employed in epitope recognition.

We have utilised fluorescence activated cell sorter (FACS) analysis to define the Vβ family employed in the recognition of T4+(2553) peptide by the CH9 TCR. The T-cell clone MTg12B (Champion *et al.,* 1985) which is the parent clone of the CH9 hybridoma was used. Cells were stained using one of three anti-mouse Vβ antibodies which were obtained from Dr A Cooke (UCMSM, UK). Using rat anti-mouse anti-Vβ 6 (44.22.1), Vβ 8.1, 2 & 3 (F23.1) & Vβ 11 (KT11), binding was detected using 1/500 rabbit anti-rat Ig FITC, the cells being double stained with a PE conjugated CD4 to ensure that the population observed was CD4+ve. Results shown in figure 3.4B panels ***a) - c)*** indicate that the TCR employed

in recognition of this epitope by MTg12B and therefore CH9 is restricted to Vβ8.1, 2 & 3. Panels ***a)*** & ***c)*** show that MTg12B is Vβ6 and Vβ 11 negative, whereas panel ***b)*** clearly shows MTg12B being positive for Vβ8.1, 2 & 3. These results are shown in comparison with D10 cells which are known to be Vβ8 positive panels ***d) -f)***. This clearly demonstrates the positivity of MTg12B for Vβ8 (1,2 &3). Figure 3.4B is shown on the next page.

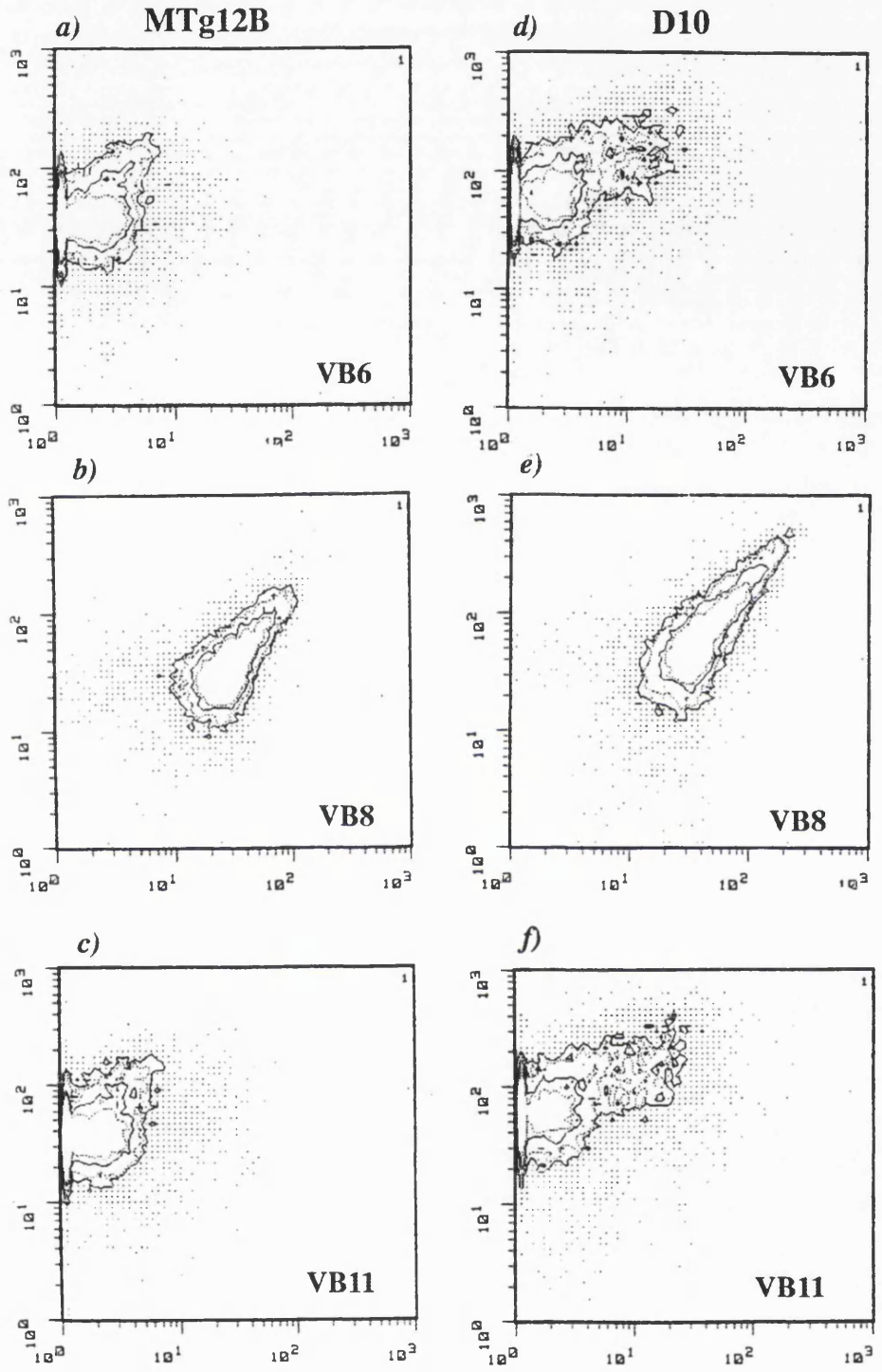


Figure:- 3.4B. A Vβ8 TCR is employed in epitope recognition.

MTg12B (parent line to CH9) cells were stained with one of four rat anti-mouse monoclonal antibodies. These were anti-Vβ6, Vβ8, Vβ11 or a non specific rat anti mouse IgG as a first layer, binding was detected using a rabbit $F(ab')_2$ anti-rat IgG-FITC. These were then counter stained using an anti-mouse monoclonal to CD4-PE conjugate to enable the CD4 population to be assessed and analysed by FACS. A cell line D10 which is known to be Vβ8 positive acted as a positive control. Panels ***a) - c)*** indicate MTg12B staining and panels ***d) - f)*** indicate D10 staining.

3.5 The effect of residue substitution on T4+(2553) peptide reactivity of CH9.

3.5A) CH9 response to substituted peptides.

In an attempt to delineate the important residues with the T4+(2553) peptide, sets of peptides based on the 11 mer stimulatory peptide structure were synthesised. Each set consisted of eight peptides for each of the eleven residues (excluding the T4 at residue 5) in which the original residue was substituted by one of eight amino acids. The substituting amino acids were as follows :- E (glutamic acid), G (glycine), K (lysine), L (leucine), N (asparagine), P (proline), R (arginine) and Y (tyrosine). The ability of these peptides to stimulate CH9 was tested in comparison to the original peptide and the results are expressed as the % inhibition of maximal response calculated as:

$$\% \text{ Inhibition} = \frac{100x\ (\text{CPM unsubstituted peptide-B}) - (\text{CPM substituted peptide-B})}{(\text{CPM unsubstituted peptide-B})}$$

Where B = background counts.

Thus negative values indicate stimulation and positive values indicate an inhibition of the control response.
Residue positions shown below:

Residue Positions:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Amino Acids	**S**	**T**	**D**	**D**	**(T4)**	**A**	**S**	**F**	**S**	**R**	**A**

Figure 3.5A shows the inhibition data obtained using these substituted peptides and the CH9 T-cell hybridoma. It is clear from the figure that substitutions at either residue one (S = serine) or two (T = threonine) have no effect on the stimulatory capacity apart from a very low level of general stimulation. For residue three the pattern is almost exactly the same except substitutions of the original residue (D = aspartic acid) for glycine, arginine or tyrosine inhibit slightly. At residue four the effect is more marked. Substitution of the aspartic acid residue for glycine and tyrosine cause a significant decrease in stimulation (>50%), substitution by lysine a smaller decrease (approx 25%). A similar pattern is seen for residue 6, substitutions by lysine and tyrosine causing more than a 50% decrease in stimulation with leucine a causing slightly smaller decrease. At residue 7, only substitution of the serine residue for tyrosine causes any marked decrease in stimulation, although a small effect is seen on substitution with an arginine residue. Substitution at residue eight, phenylalanine, considerably diminishes the stimulatory capacity of the peptide. The only substitution which maintains this capacity completely is arginine while substitutions by lysine and asparagine only slightly diminish the stimulatory capacity of the peptide. Tyrosine causes a 40% drop in stimulatory capacity but the effect is very marked when phenylalanine is substituted by glutamic acid, glycine, lysine or proline when the inhibition

of stimulatory capacity is > 90%. In comparison to residue eight, residue nine is almost completely inert except for the substitution of serine for tyrosine which causes a 50% diminution in peptide stimulatory capacity. Residue ten (arginine) is again very important; one of the substituting amino acids (arginine) is the actual amino acid in the original peptide and therefore as expected maintains maximal stimulatory capacity, all other substitutions diminish stimulatory capacity, two of which do so by more than 90% (leucine and asparagine). Finally residue eleven (alanine) appears to be moderately reactive. Substitutions by leucine, asparagine or tyrosine cause a moderate decrease in stimulatory capacity (<25%) whereas substitutions by glutamic acid or arginine cause an 80-90% decrease in stimulatory capacity of the un-substituted 11 mer peptide. Figure 3.5A is shown on the next four pages.

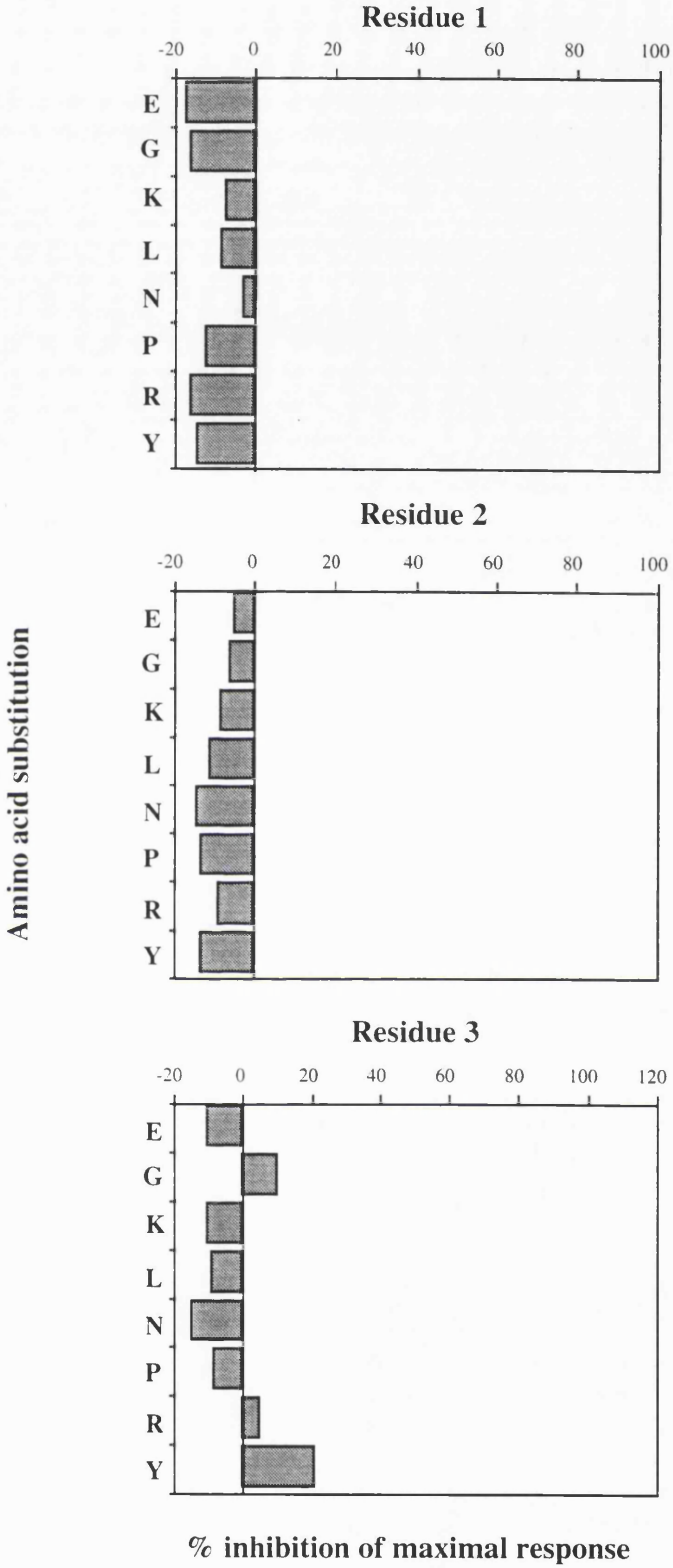
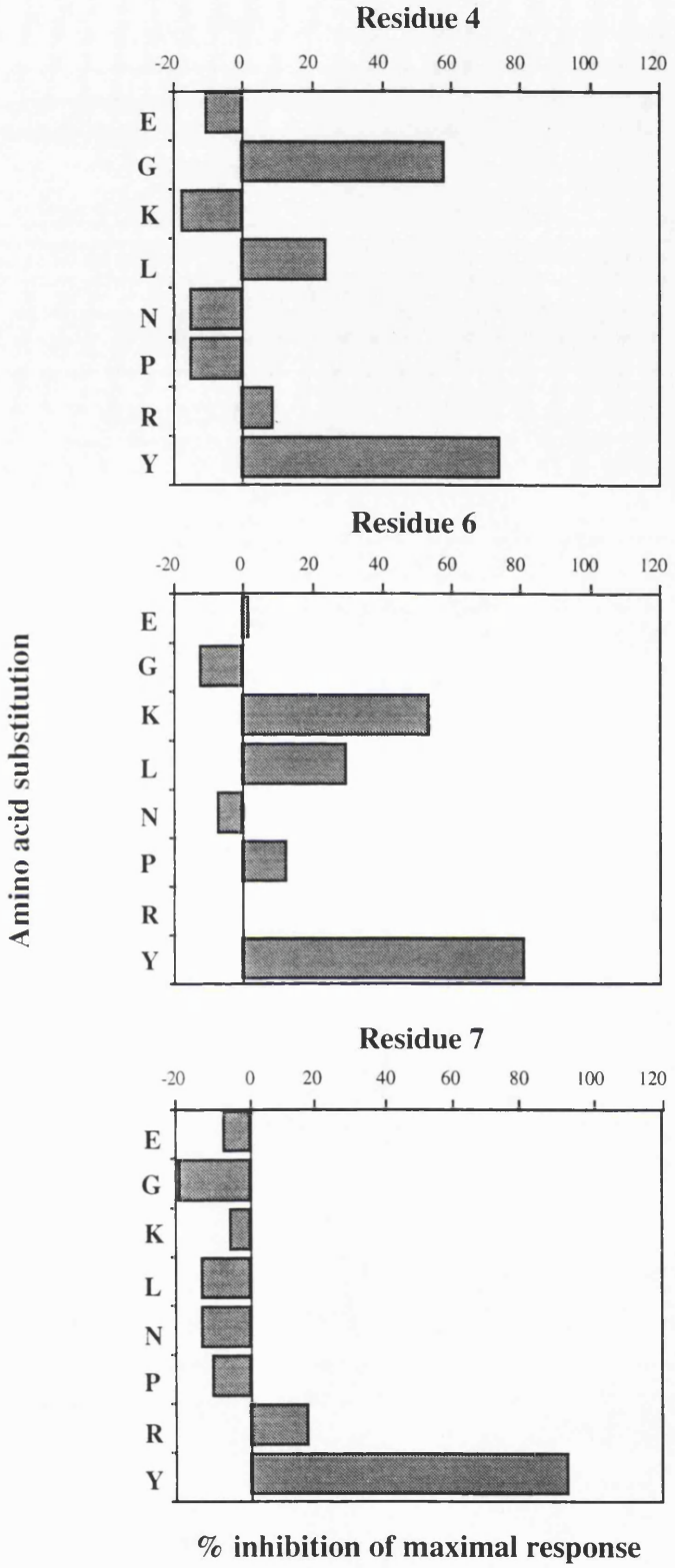


Figure:- 3.5A. Effect of residue substitution on peptide reactivity.

Response of $2x10^5$ CH9 to 2.5 µg/ml 11 mer peptide STDD(**T4**)ASFSRA substituted at all positions except the T4 with each of the following amino acids E,G,K,L,N,P,R &Y. Results are expressed as the % inhibition of the maximal response to unsubstituted peptide by each substitution at each residue. Supernatants taken 24h, for assessment of IL-2 release, results shown are average of triplicate cultures. Results shown are the average of four separate experiments.



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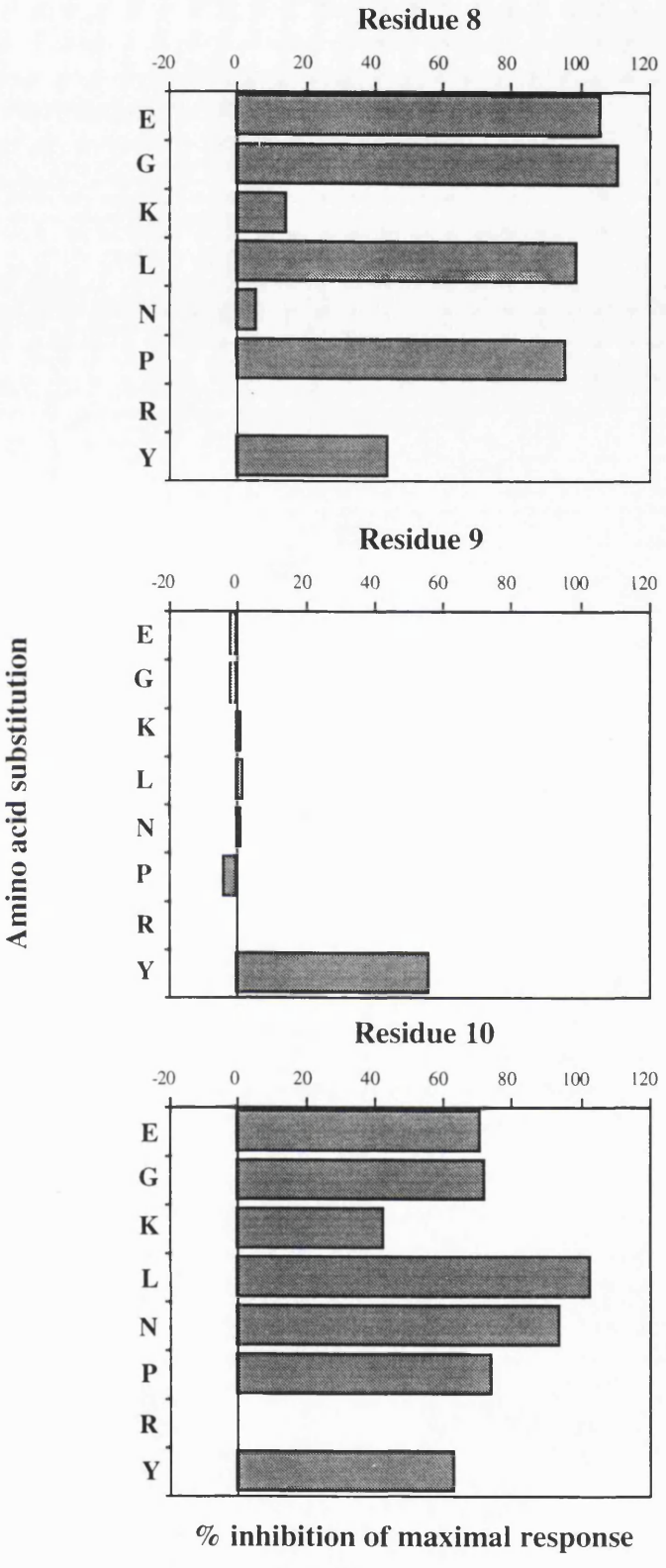

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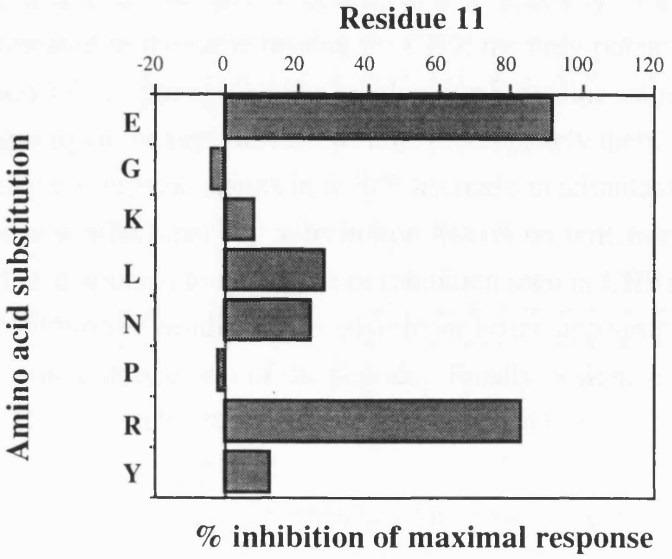

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Response of $2x10^5$ CH9 to 2.5 µg/ml 11 mer peptide STDD(**T4**)ASFSRA substituted at all positions except the T4 with each of the following amino acids E,G,K,L,N,P,R &Y. Results are expressed as the % inhibition of the maximal response to unsubstituted peptide by each substitution at each residue. Supernatants taken 24h, for assessment of IL-2 release, results shown are average of triplicate cultures. Results shown are the average of four separate experiments.

3.5B) ADA2 response to substituted peptides.

A parallel set of results have been obtained for the cell line ADA2 (figure 3.5B). This T-cell hybridoma line has been shown to be clonotypically distinct from CH9 (Rayner *et al.*, 1987), is also H-$2A^k$ restricted and been shown to respond to the same thyroxine residue in thyroglobulin as CH9 (Champion *et al.*, 1992) but the pattern of response is slightly different. From figure 3.5B it appears that the substitution of residues one to four has little affect on the inhibition of stimulation by the peptide of the ADA2 clone. However, certain amino acid changes in residues one and two appear to be able to increases the stimulation of the peptide in comparison to the original unsubstituted peptide. In this data set there appears to be a small degree of internal variation between -20 and +20, these results must not be discounted but they may just reflect the degree of error as a result of only being able to average two experiments. The previous set of data for the clone CH9 are the average of four separate experiments, hence the degree of variation around the zero mark is smaller.

At residue one, the only substitution that has an effect, in that it increases the peptide stimulatory capacity, is the substitution of serine for lysine. Exactly the same effect is seen or residue two where the substitution of threonine for lysine leads to an increase in the stimulatory capacity of the peptide. Residues six (alanine) when substituted by the amino acids glutamic acid, lysine or leucine results in a 50% decrease in the stimulatory capacity of the peptide compared to the unsubstituted control. For residue 7, proline is

the only substitution that is able to reduce peptide reactivity. Residue eight is virtually inert when compared to the same residue for CH9; the only potential effect appears to be the substitution of the phenylalanine residue for a proline causes a small increase in stimulatory capacity of the peptide, residue nine is completely inert. In residue ten, arginine when substituted by tyrosine results in a 70% decrease in stimulatory capacity of peptide. This is the greatest effect that any substitution has on presentation of peptide to ADA2 , but is small when compared to the degree of inhibition seen in CH9 responses to substituted peptides. Substitution of residue ten by glycine or lysine appears to have a small effect by increasing the stimulatory effect of the peptide. Finally, residue eleven, alanine, displays little activity. The only substitution that results in any degree of inhibition of stimulation is that of alanine for glutamic acid. On the whole the reactivity of substituted peptides when compared to the pathogenic peptide are far less than seen for the counterpart clone, CH9. Figure 3.5B is shown on the next four pages.

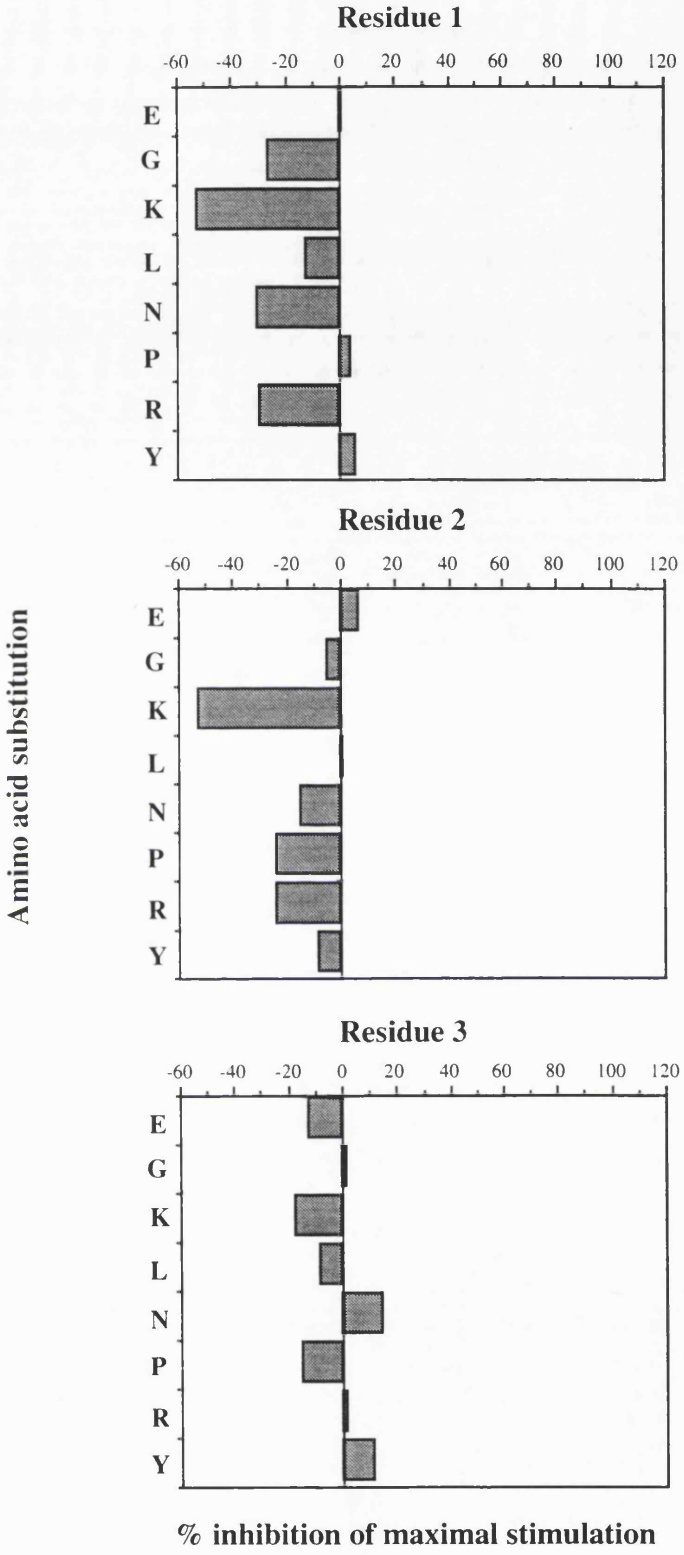


Figure:- 3.5B. Effect of residue substitution on peptide reactivity.

Response of $2x10^5$ ADA2 to 2.5 µg/ml 11 mer peptide STDD(**T4**)ASFSRA substituted at all positions except the T4 with each of the following amino acids E,G,K,L,N,P,R &Y. Results are expressed as the % inhibition of the maximal response to unsubstituted peptide by each substitution at each residue. Supernatants taken 24h, for assessment of IL-2 release, results shown are average of triplicate cultures. Results shown are the average of two separate experiments.

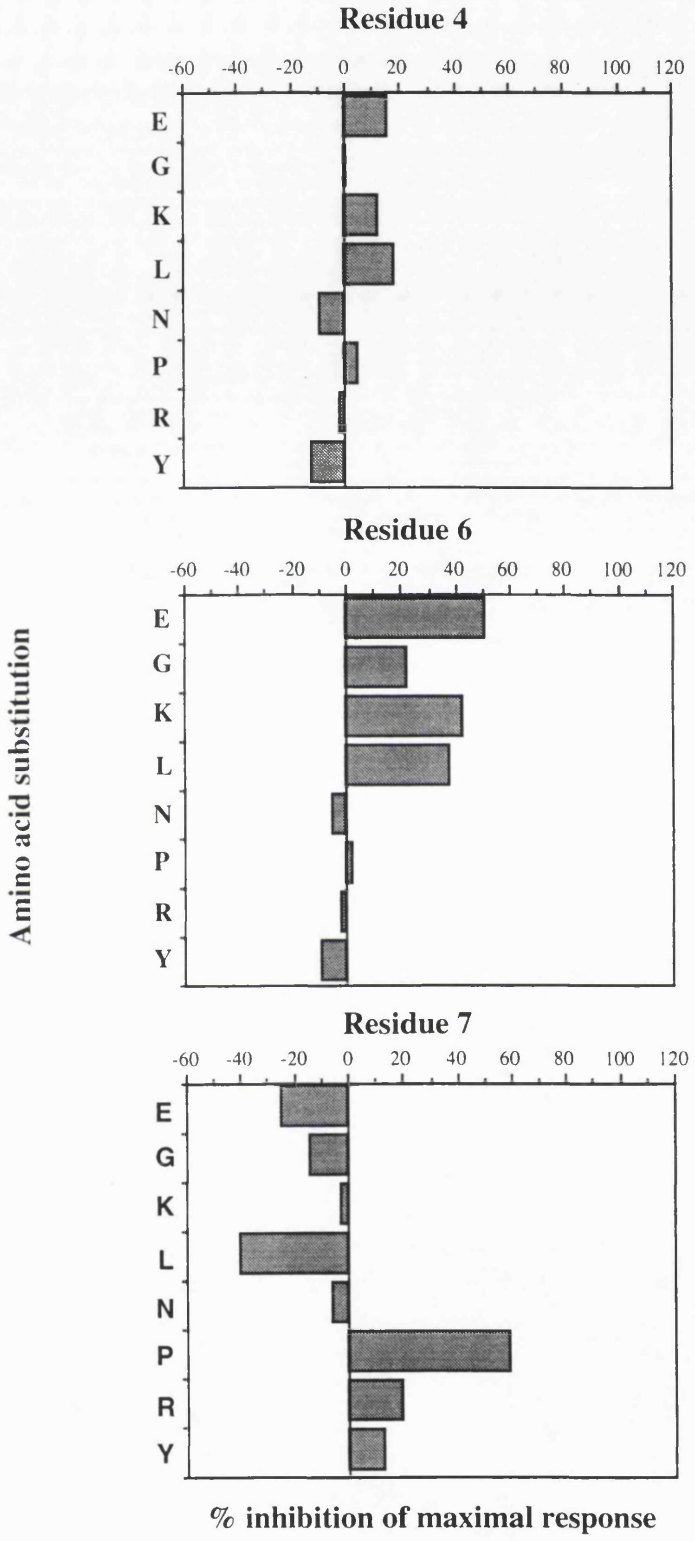


Figure:- 3.5B. Effect of residue substitution on peptide reactivity.

Response of $2x10^5$ ADA2 to 2.5 µg/ml 11 mer peptide STDD(**T4**)ASFSRA substituted at all positions except the T4 with each of the following amino acids E,G,K,L,N,P,R &Y. Results are expressed as the % inhibition of the maximal response to unsubstituted peptide by each substitution at each residue. Supernatants taken 24h, for assessment of IL-2 release, results shown are average of triplicate cultures. Results shown are the average of two separate experiments.

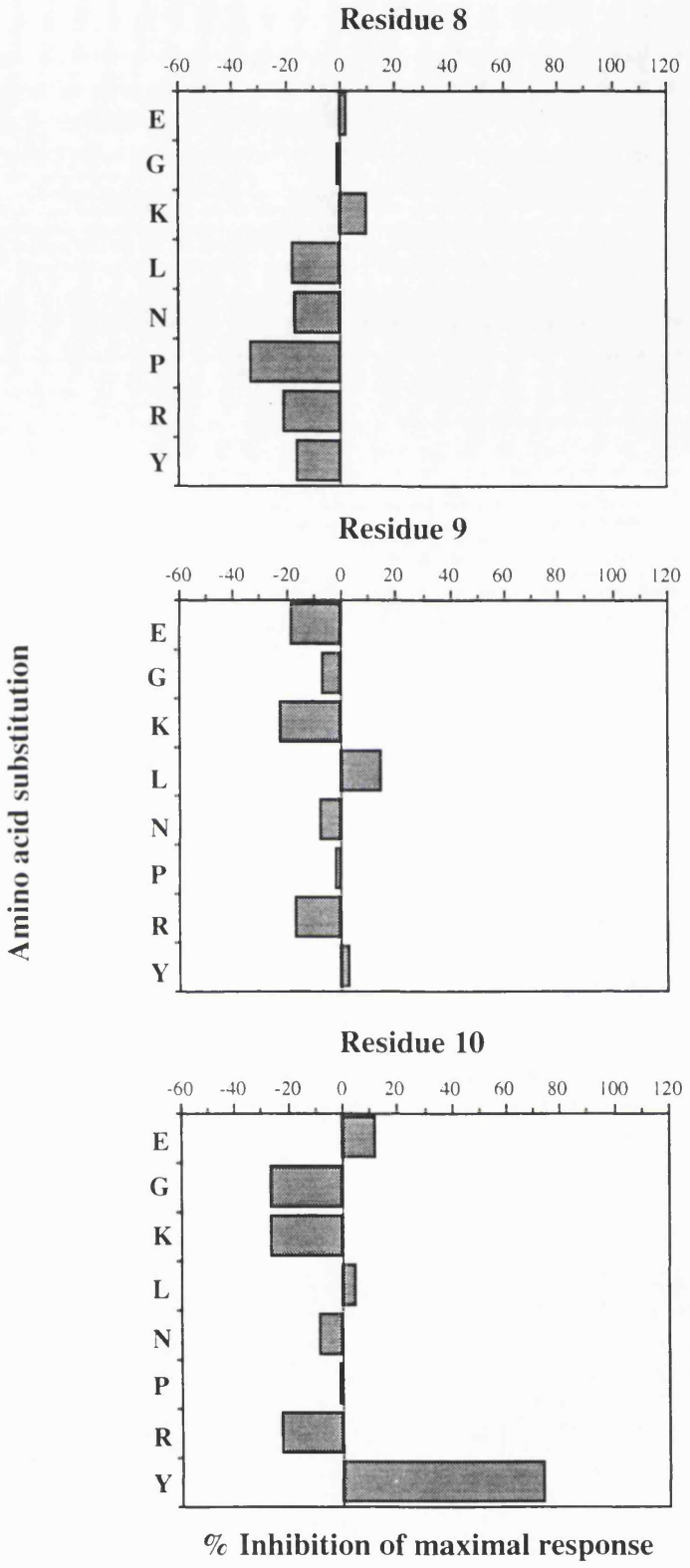


Figure:- 3.5B. Effect of residue substitution on peptide reactivity.

Response of $2x10^5$ ADA2 to 2.5 µg/ml 11 mer peptide STDD(**T4**)ASFSRA substituted at all positions except the T4 with each of the following amino acids E,G,K,L,N,P,R &Y. Results are expressed as the % inhibition of the maximal response to unsubstituted peptide by each substitution at each residue. Supernatants taken 24h, for assessment of IL-2 release, results shown are average of triplicate cultures. Results shown are the average of two separate experiments.

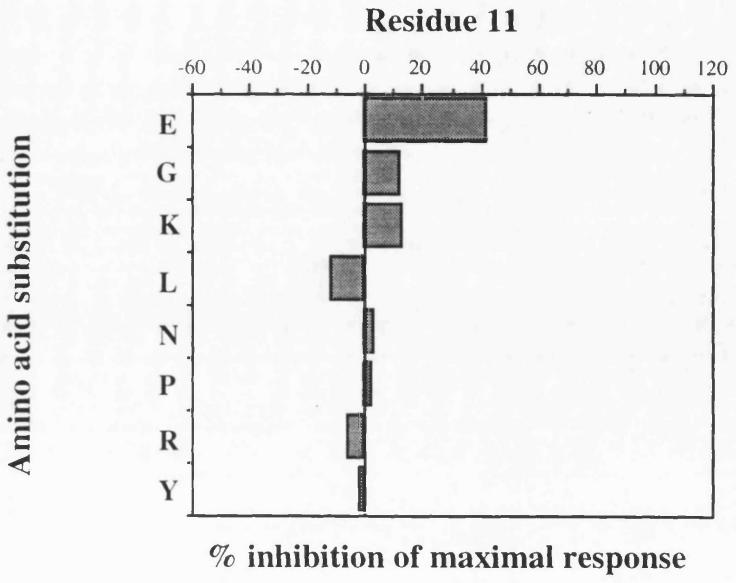


Figure:- 3.5B. Effect of residue substitution on peptide reactivity.

Response of $2x10^5$ ADA2 to 2.5 µg/ml 11 mer peptide STDD(**T4**)ASFSRA substituted at all positions except the T4 with each of the following amino acids E,G,K,L,N,P,R &Y. Results are expressed as the % inhibition of the maximal response to unsubstituted peptide by each substitution at each residue. Supernatants taken 24h, for assessment of IL-2 release, results shown are average of triplicate cultures. Results shown are the average of two separate experiments.

4.0 Analysis of the *in vivo* T-cell epitope .

We have been able to identify a particular iodinated T-cell epitope that is recognised by autoreactive T-cells *in vitro*, but the verification of the potential role for this epitope in a disease situation can only come from looking at the response *in vivo* and whether it is able to induce disease similar to that induced by the whole molecule.

4.1 Thyroxine containing T4+(2553) peptide is recognised by MTg primed lymph node cells.

Draining lymph nodes were taken on day 8 following immunisation of CBA/J (H-2^k) susceptible strain mice with 50µg normal outbred strain mouse thyroglobulin in CFA once in the hind footpads on day 0. Figure 4.1 panel ***a)*** shows that mice that were immunised with MTg made a dose dependent response when challenged with MTg *in vitro*, but also responded to the 11 mer T4+(2553) peptide. In contrast, when the mice are immunised using T4+(2553) peptide (panel ***b***), there was no response to MTg but a good response to T4+(2553) peptide at all doses used *in vitro*. Thus the thyroxine containing peptide T4+(2553) previously identified is produced by the processing of MTg and recognised *in vivo*.

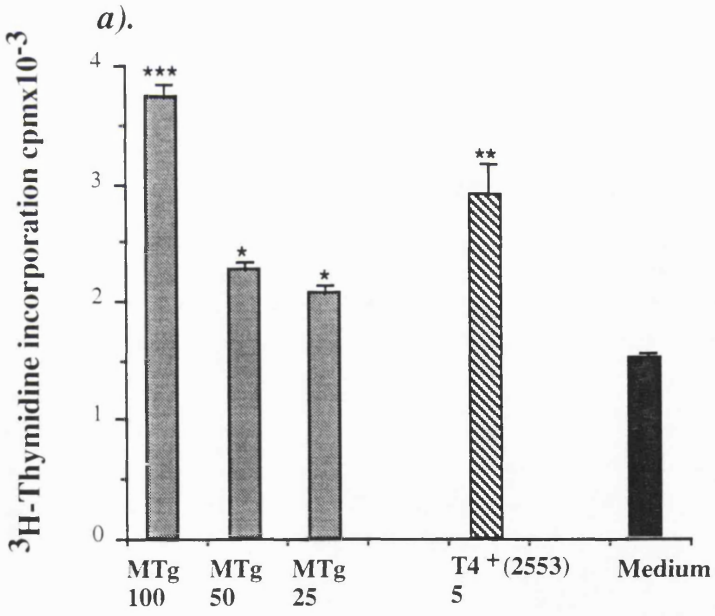


Figure:- 4.1. Thyroxine-containing T4+(2553) peptide is recognised by MTg primed lymph node cells.

Response of 2x10^5 Day 8 LNC immunised with ***(a)*** 50µg MTg/CFA cultured in the presence of MTg (-□-) or T4+(2553) peptide (-▨-). Medium control is shown (-■-). Proliferations were assessed by (^{3}H)-Thymidine incorporation and results shown are average of triplicate cultures +/- SEM. (Non significant values not indicated, * $p<0.05$, ** $p<0.01$ and ***$p<0.001$).

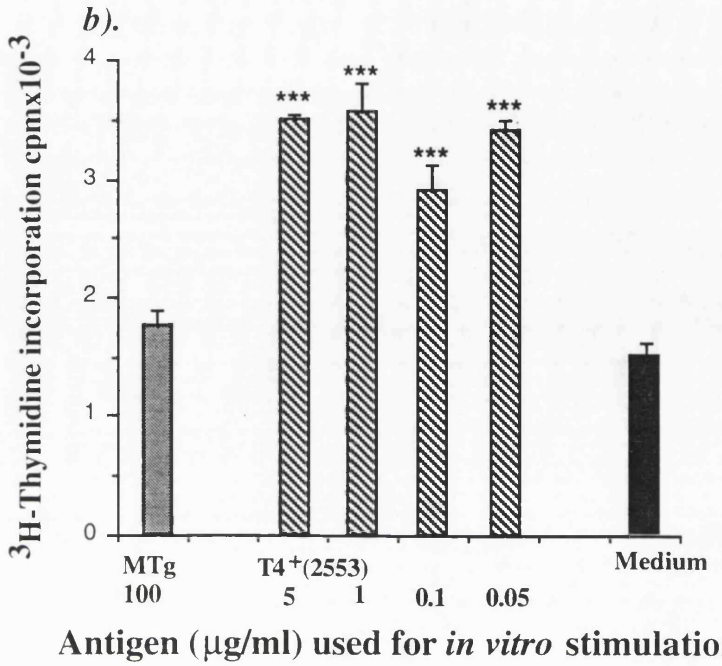


Figure:- 4.1. Thyroxine-containing T4$^+$(2553) peptide is recognised by MTg primed lymph node cells.

Response of 2x10^5 Day 8 LNC immunised with ***(b)*** 5μg T4$^+$(2553) peptide cultured in the presence of MTg (-▒-) or T4$^+$(2553) peptide (-▨-). Medium control is shown (-■-). Proliferations were assessed by (^{3}H)-Thymidine incorporation and results shown are average of triplicate cultures +/- SEM. (Non significant values not indicated, * $p<0.05$, ** $p<0.01$ and ***$p<0.001$).

4.2 Non-iodinated Tg (ATA-Tg) priming does not prime for responses to the T4$^+$(2553) peptide.

Figure 4.2 panel ***a)*** shows the responses of MTg primed LNC to stimulation *in vitro* by MTg or T4$^+$(2553) peptide. Although the responses are low, there is clearly a response to 50μg/ml MTg and a dose response to the T4$^+$(2553) peptide, whereas there is no response to T4$^-$(2553) peptide. This is in contrast to the comparable responses seen following ATA-Tg priming ***b)***, in which there appears to be no response to T4$^+$ (2553) peptide whilst there is a small response to MTg. LNC from CBA/J mice which have been primed with T4$^+$(2553) peptide do not respond to MTg *in vitro* but make good responses to T4$^+$(2553) ***c)*** peptide in a dose-dependent manner. This confirms that MTg priming, is also able to prime for T4$^+$(2553) peptide and that this peptide only seems to prime for peptide responses. On the other hand, non-iodinated thyroglobulin (ATA-Tg) is unable to prime for the T4$^+$(2553) peptide although there may be some small response to MTg. This lack of responsiveness is presumably due to the lack of iodinated epitopes on ATA thyroglobulin.

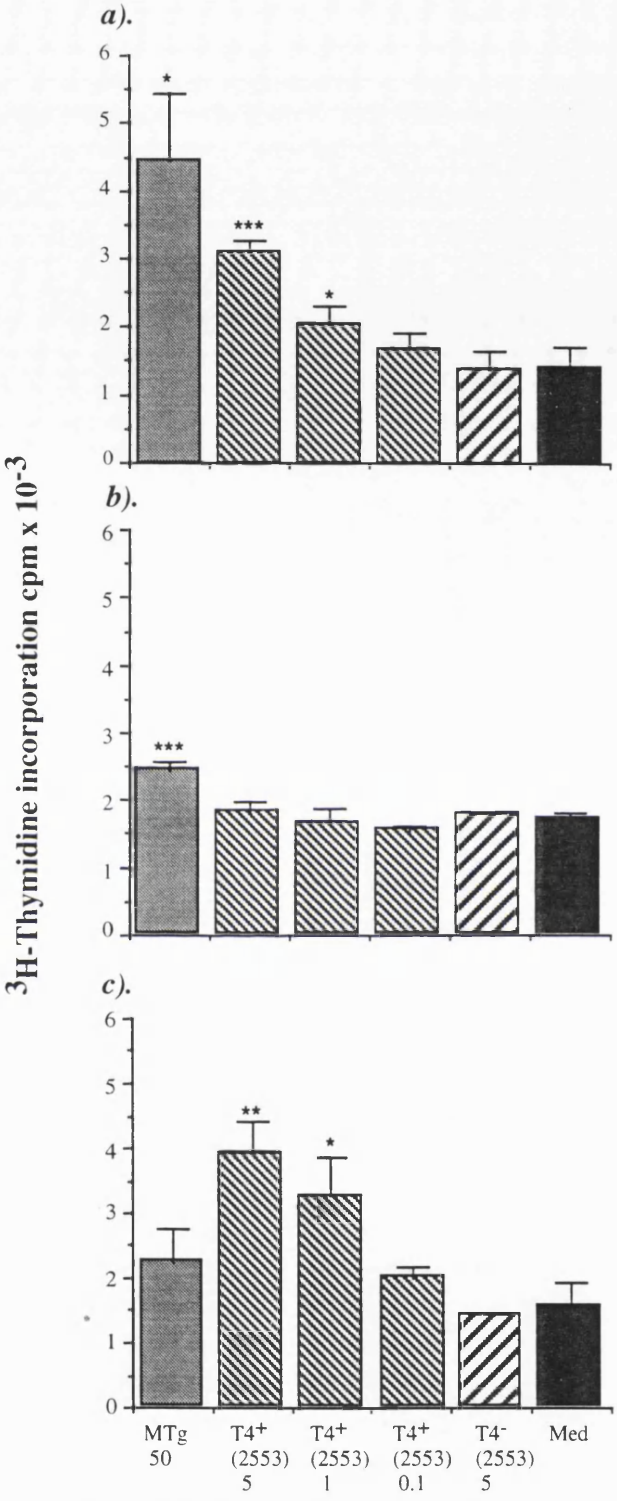


Figure:- 4.2. ATA-Tg priming does not prime for responses to the T4$^+$(2553) peptide.
Response of 2x10^5 Day 16 LNC immunised with *(a)* 50µg MTg/CFA, *(b)* 50µg ATA-Tg/CFA or *c)* 5µg T4$^+$(2553) peptide cultured in the presence of MTg (-▨-), T4$^+$(2553) peptide (-▧-) or T4$^-$(2553) peptide (-▨-). Medium control is shown (-■-). Proliferations were assessed by (^{3}H)-thymidine incorporation and results shown are average of triplicate cultures +/- SEM. (Non significant values not indicated * $p<0.05$, ** $p<0.01$ and ***$p<0.001$).

4.3 Anti-CD4 therapy when priming with MTg can tolerise responses to $T4^+$(2553) peptide.

Treatment with anti-CD4 monoclonal antibody has been shown to have a beneficial effect in experimental autoimmune disease, in that anti-CD4 has been shown to prevent the induction of EAT by depletion of the CD4 +ve T-cell population (Hutchings *et al.*, 1993, Kong *et al.*, 1989a).

In contrast to this anti-CD8 has little effect in prevention of infiltration of the thyroid in the experimental system. Therefore it was considered of interest to see whether non-depleting anti-CD4 was able to prevent the induction of EAT by thyroglobulin either by blocking the CD4 cells or inducing a specific tolerance. It was also of interest to see whether tolerisation to Tg induced a concomitant tolerance to the thyroxine containing peptide $T4^+$(2553).

Figure 4.3 panel ***a)*** shows the *in vitro* responses of MTg primed lymph node cells taken 16 days after priming with MTg in the presence and absence of anti-CD4. In the absence of anti-CD4 the normal responses to MTg and peptide *in vitro* are seen. However in the presence of anti-CD4, these *in vitro* responses are completely abrogated. The control immunisation was conducted using PBS/CFA, shown in panel ***b)*** where it can be seen that no immune responses are mounted against the peptide unless there is specific antigenic immunisation, the only response is to the T-cell mitogen Con A. This non-specific response is not down regulated by anti-CD4 treatment but rather is potentiated. Figure 4.3 is shown on the next page.

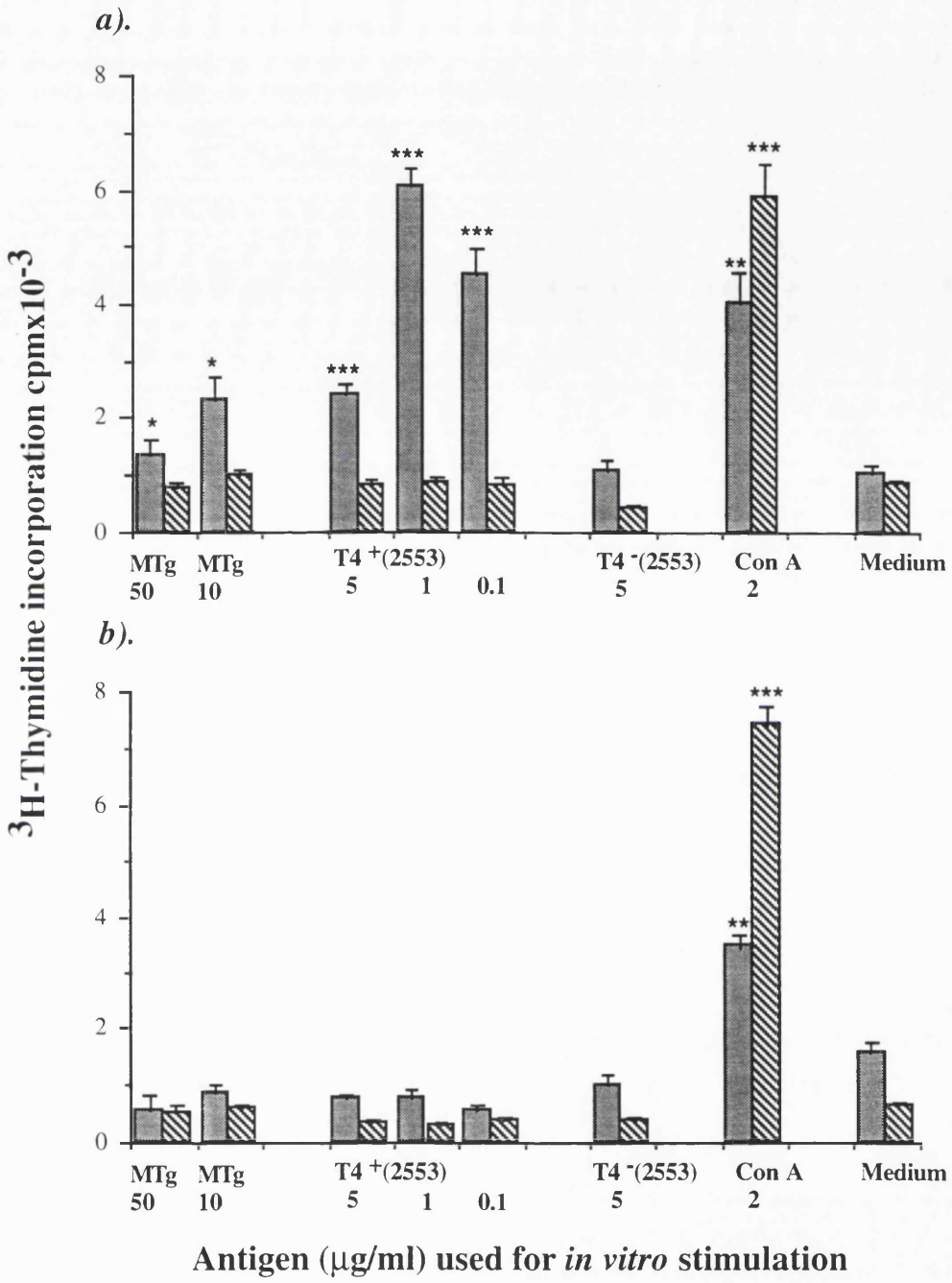


Figure:- 4.3. Anti-CD4 therapy when priming with MTg can tolerise to the T4$^+$(2553) peptide.

Response of 2x10^5 Day 16 LNC immunised with ***(a)*** 50μg MTg/CFA plus (-▨-) or minus (-□-) non-depleting anti-CD4 or ***(b)*** PBS/CFA plus (-▨-) or minus (-□-) non-depleting anti-CD4 cultured in the presence of MTg, T4$^+$(2553) peptide or T4$^-$(2553) peptide, the Con A and medium controls are shown as indicated on the graph. Proliferations were assessed by (^{3}H)-thymidine incorporation and results shown are average of triplicate cultures +/- SEM. (Non significant values not indicated * $p<0.05$, ** $p<0.01$ and ***$p<0.001$).

4.4 Analysis of the epitopes recognised in other susceptible strain mice.

It has been shown that the T4+(2553) peptide is processed and presented correctly from thyroglobulin in susceptible strain mice such as the CBA/Ca and CBA/J which are both of the H-2^k haplotype. Other strains such as H-2^q and H-2^s are also susceptible to the induction of EAT by the administration of thyroglobulin in CFA, two examples of these haplotypes being the DBA/1 (H-2^q) and the SJL (H-2^s). To see whether similar epitopes are recognised in these other strains, animals were immunised with MTg/CFA in the hind footpads with and the popliteal lymph nodes taken on day 8. The cells were then used in a lymph node restimulation assay with a set of peptides which covered the four hormonogenic sites on human and mouse thyroglobulin. The peptides used were 11 mers with the following sequences: NIFE(T4)QVDAQP (psn 5), STDD(T4)ASFSRA (psn 2553), ATRD(T4)FIICPI (psn 2567) & LQEPGSKT(T4)SK (psn 2746) at a concentration of at 10, 5, 1 & 0.5 µg/ml. Figure 4.4 shows the responses of the cells from the various haplotype mice, panels *a)* and *b)* indicate the responses obtained from the H-2^k haplotype. The CBA/Ca strain, although it is a susceptible haplotype, is a low responder for induction of thyroiditis. From the data shown in figure 4.4 panel *a)* it can be seen that there are T-cells which recognise the epitope at position 2553 following immunisation by MTg. This is the same position as that recognised by CH9 and ADA2 which are CBA/Ca derived hybridoma lines. Panel *b)* shows the responses obtained for the CBA/J mouse which is also H-2^k but is a high responder. This also recognises the same epitope as CBA/Ca and in the CBA/J mouse this epitope has been shown to be pathogenic (Hutchings *et al.*, 1992). Panel *c)* shows the responses obtained in the SJL (H-2^s) mouse which is also susceptible to the induction of EAT, there do not appear to be any responses to any of the T4 regions. This is in contrast to the DBA/1 (H-2^q) strain shown in panel *d)*. This clearly makes some response to peptide covering position 5 on the thyroglobulin molecule although it does not appear to be as strong as the responses seen in the H-2^k haplotype. Therefore, this is evidence that there are epitopes that are haplotype specific within the murine population. Figure 4.4 is shown on the next two pages.

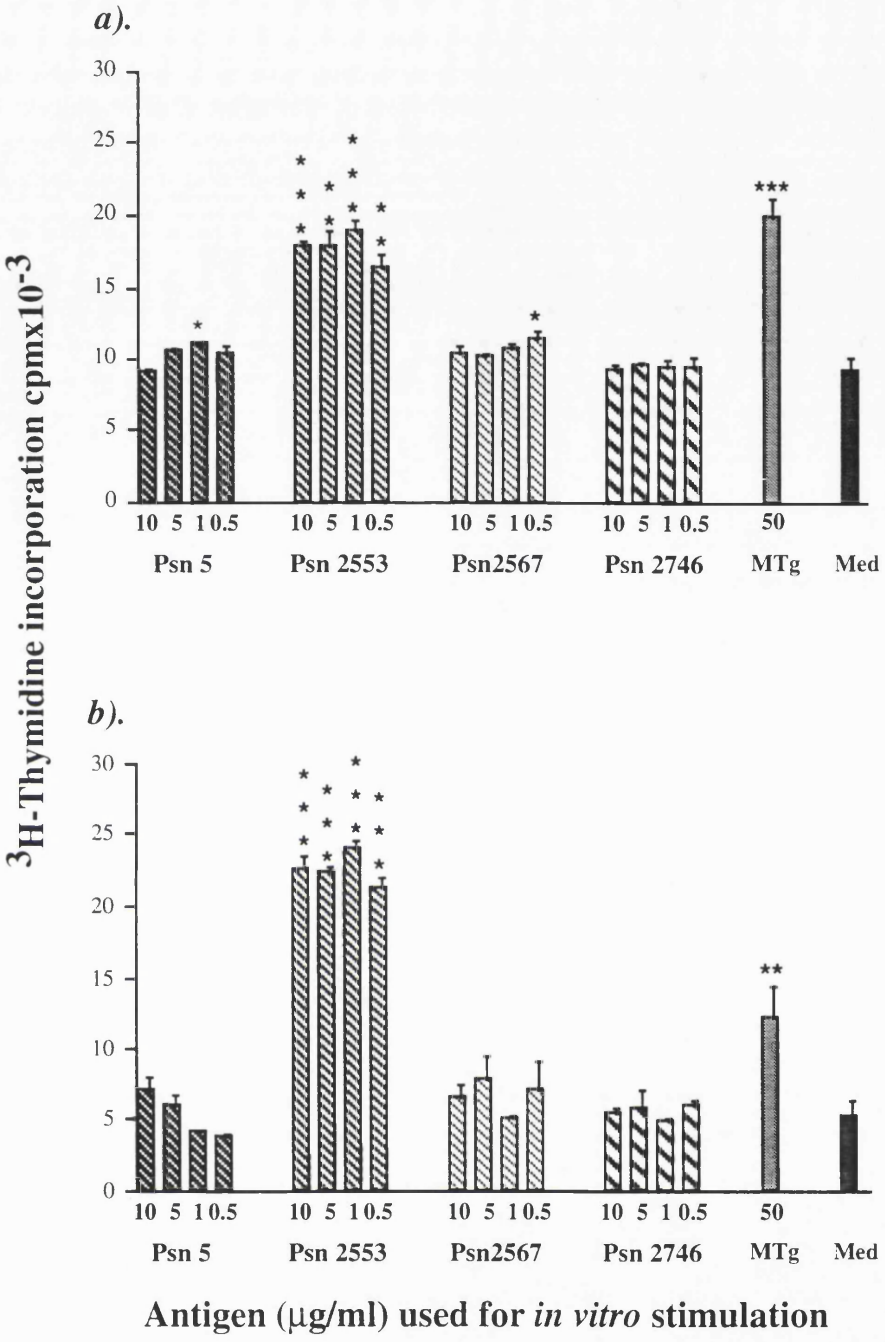
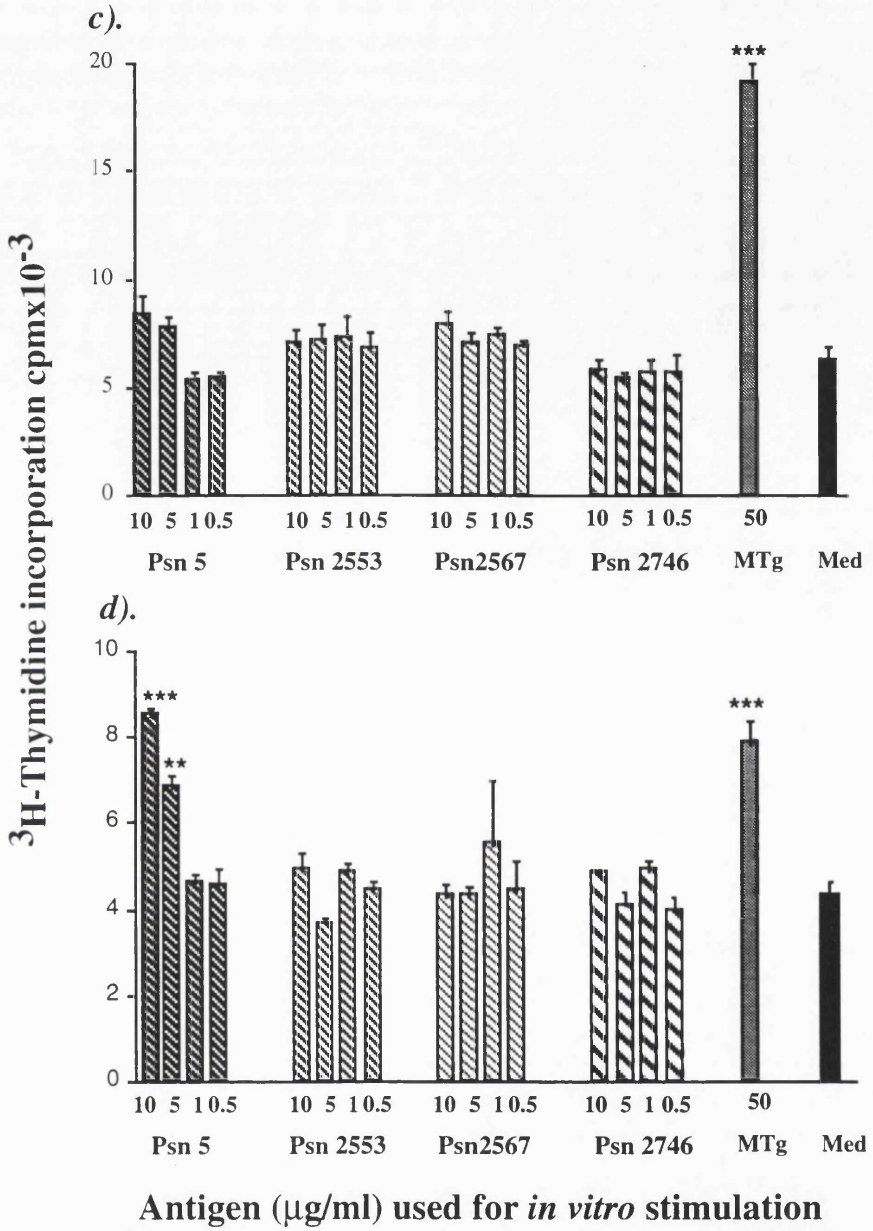


Figure:- 4.4. Analysis of the epitopes recognised in other susceptible strain mice.
Response of 2x10^5 Day 16 LNC immunised with 50µg MTg/CFA cultured in the presence of 11 mer peptides representing the four hormonogenic sites found in thyroglobulin. Dose responses of susceptible strain LNC to peptide from position 5 (-▨-), 2553 (-▨-), 2567 (-▨ -) and 2745 (-▨-) and control responses to 50 µg MTg (-▩-) and medium (-■-) are shown in each of the four panels. CBA/Ca responses are shown in *(a)*, CBA/J *(b)*, Proliferations were assessed by (^{3}H)-thymidine incorporation and results shown are average of triplicate cultures +/- SEM. (Non significant values not indicated * $p<0.05$, ** $p<0.01$ and ***$p<0.001$).



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5.0 Antigen presentation by thyrocytes.

The *in vivo* evidence for presentation of the $T4^+$(2553) peptide to pathogenic T-cells by syngeneic splenic antigen presenting cells has been shown. However, in a human disease situation it is unlikely that APC's in the secondary lymphoid tissue will be the only cells presenting the pathogenic antigen. It has been shown that epithelial cells and in particular, human thyroid epithelial cells can be induced to express class II (Pujol-Borrell *et al.*, 1983), and that human thyroid cells taken from Hashimoto's patients also express class II (Hanafusa *et al.*, 1983) and that these cells can stimulate intrathyroidal lymphocytes in culture. It is not known at this stage whether the expression of class II and subsequent presentation of sequestered thyroid antigens is causative of, or a consequence of disease although the balance of evidence suggests that IFN-γ expression is secondary to thyroidal infiltration. We have looked for evidence of the thyroid as a potential APC in the murine EAT system.

5.1 Determination of optimal conditions for growth and assay function.

5.1A) The effect of culture period.

Thyrocytes were initially cultured according to two different protocols to determine which was most suitable to assess presentation to Tg specific hybridomas. The first method was that used by Yeni & Charreire (1981), where thyrocytes were used as antigen presenting cells from day five of culture and cultured in a simplified basal medium. The method previously used by workers in the Department of Immunology used thyrocytes from day 12 of culture and a complicated, hormone supplemented growth medium. These two methods were compared using the basal medium described by Yeni & Charreire (1981). Figure 5.1A shows the levels of presentation achieved using these two time points. Panel *a)* shows levels of presentation to CH9 by CBA/Ca thyroid epithelial cells cultured for five days before use. It can be seen that in these experiments mouse thyroglobulin is not processed and presented by the TEC's in the presence or absence of IFN-γ. This is in contrast to the 11 mer $T4^+$(2553) peptide which is presented by the TEC's in the absence of IFN-γ, this may indicate the presence of a small amount of class II already present on the cell surface. However this effect is substantially up-regulated by the addition of 100 U/ml IFN-γ to the culture medium for 72 h prior to assay.

Thyroid epithelial cells are able to synthesise Tg in culture and therefore might have been able to present endogenously derived peptide by the class II pathway. Given the requirement for iodinated Tg for the induction and recognition of the pathogenic epitope, sodium iodide (NaI) at a final concentration of 1μM was incorporated into the culture medium to ensure that if epitopes were processed they would be fully iodinated and therefore be recognised. In this experiment there appears to be a small degree of endogenous presentation in the presence of NaI which is statistically significant. In other experiments no endogenous presentation was seen, thus this phenomenon seems to be unreproducible. Although the level of endogenous presentation was statistically significant it is probably not biologically

significant. Figure 5.1B panel *a)* shows an example of a number of experiments which gave the same result, however there were other experiments that exhibited the same pattern of results as in *b)* therefore the results at 12 days did not appear to be very consistent. This may have been due to the long culture time allowing the outgrowth of contaminating cells such as fibroblasts. No quantification was made as to the comparative levels of contamination between five and twelve day culture, although by viewing, it could be seen that there were indeed more contaminating fibroblastic cells after twelve days than at five.

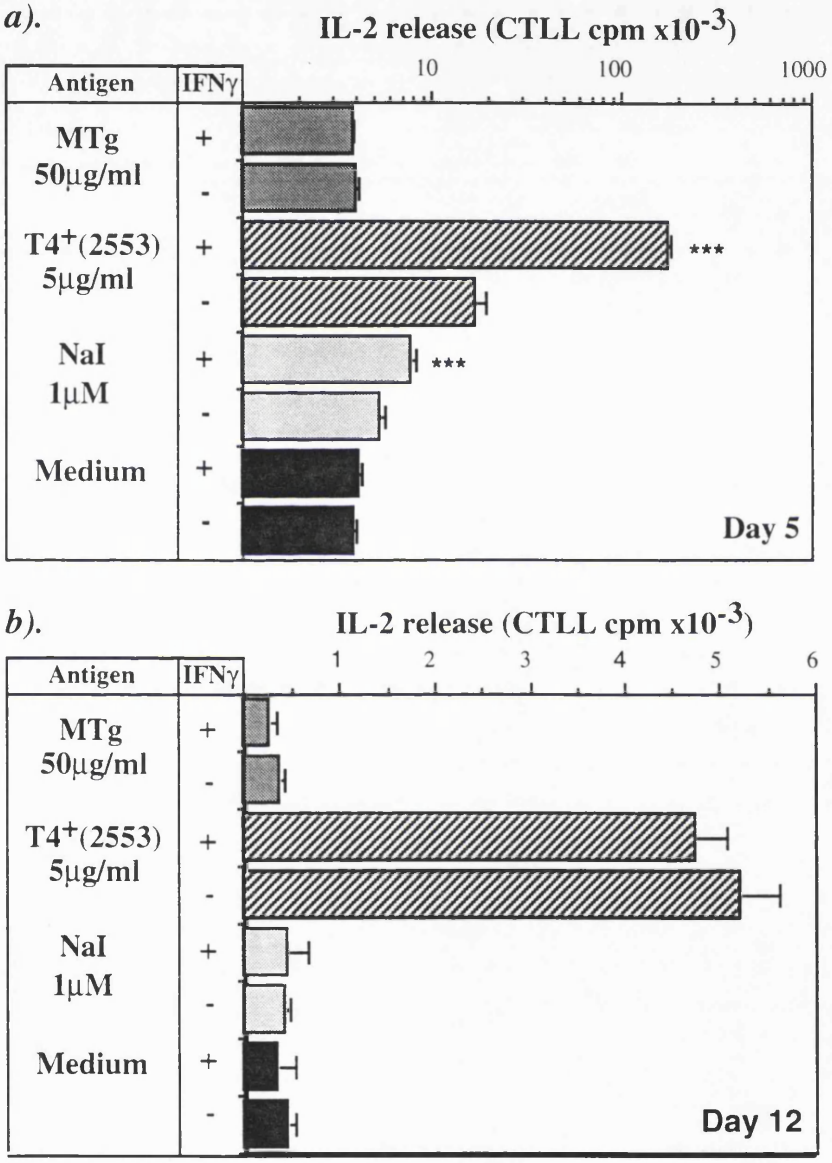


Figure:- 5.1A. The effect of culture period.

Response of $2x10^5$ CH9 to antigenic presentation by CBA/Ca thyroid epithelial cell cultures in the presence or absence of IFN-γ as indicated in the figure. Responses to 50µg MTg (-□-), 5 µg T4+(2553) peptide (-▨-), 1µM NaI (-□-) and medium control (-■-) are shown. *(a)* shows the effect of culture of TEC for 5 days prior to assay and *(b)* shows the effect of culture of TEC for 12 days prior to assay. Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM. (Non significant values not indicated * $p<0.05$, ** $p<0.01$ and ***$p<0.001$). See Appendix 1.

5.1B) The effect of culture medium.

The two different growth mediums (basal and hormonal supplemented) were compared using the five day culture period. Figure 3.3B shows that the growth medium had no effect on the levels of presentation by the TEC's of the T4+(2553) peptide in the presence or absence of IFN-γ. Both culture mediums maintain the differential in presentation seen on the addition of IFN-γ and there is no presentation of exogenous or endogenous thyroglobulin. Therefore it was decided to use the simplified growth medium and the five day culture period as employed by Yeni & Charreire (1981) which will be referred to TEC RPMI 5%.

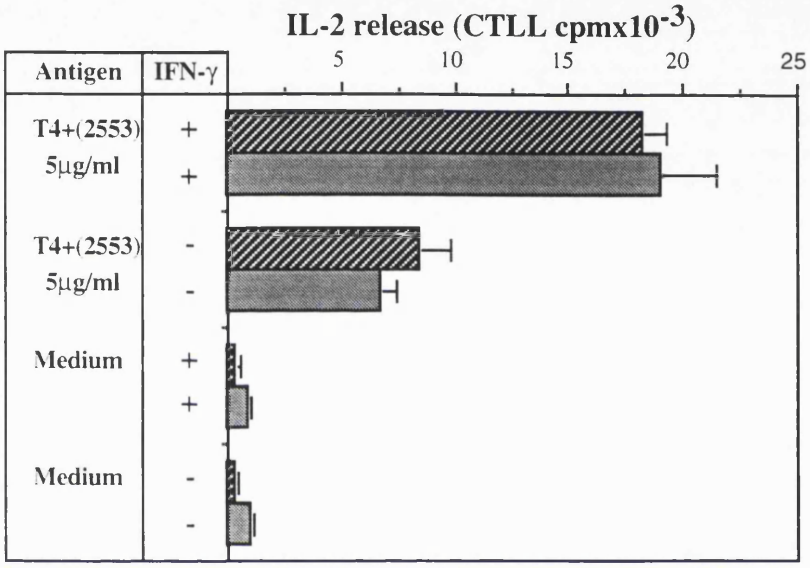


Figure:- 5.1B. The effect of culture medium.

Response of $2x10^5$ CH9 to antigenic presentation by 5 day CBA/Ca thyroid epithelial cell cultures in the presence or absence of IFN-γ as indicated in the figure. Responses to 50μg MTg, 5 μg T4+(2553) peptide, 1μM NaI and medium control are as indicated in the graph. The effect of culture medium TEC 5% (-▧-) and TEC 5% supplemented with hormones (-□-) as indicated in section 2.2.3 are shown. Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM. See Appendix 2.

5.2 The presence of ATA does not improve thyroid recognition.

It was postulated that any increases in endogenous presentation that may have been occurring was masked by high control levels of presentation as a result of iodinated thyroglobulin being present in the cells at the time of culture. If this could be removed prior to culture any newly synthesised Tg and subsequent presentation of endogenous peptide would be seen, particularly if NaI was added to the culture medium to allow any endogenously produced Tg to be fully iodinated. Initially thyroids were taken from ATA treated mice which although still making Tg, would be iodine depleted. However it proved impossible to prepare thyroid follicles from these thyroids due to the extensive hyperplasia in the glands. Even on minimal digestion, glands were totally digested resulting in a single cell

suspension containing a small number of follicles. These follicles on plating did not adhere as did normal follicles and therefore did not spread to form a monolayer. Therefore it was decided to take normal mouse thyroid glands and treat them from day two after setting up of the cultures with a 1% solution of ATA in the culture medium with the view that newly synthesised Tg may be iodine deficient unless 1μM NaI was added to the culture medium at the same time.

Figure 5.2 shows the effect of adding ATA to the culture medium. In panel ***a)***, the presence of ATA does not facilitate the detection of peptide processed from exogenously or endogenously derived peptide and levels of proliferation are not significantly elevated above background. However the presence of ATA appears to elevate the levels of $T4^+$(2553) peptide that are presented in the presence of IFN-γ. This is in contrast to other experimental results that show that the presence of ATA has no effect on the levels of $T4^+$(2553) peptide presentation in the presence of IFN-γ as shown in panel ***b)***. There is no significant increase in the levels of presentation of endogenous or exogenous Tg. Thus the presence of ATA appears not to have any obvious beneficial effect and was therefore not used in routine thyroid cell culture.

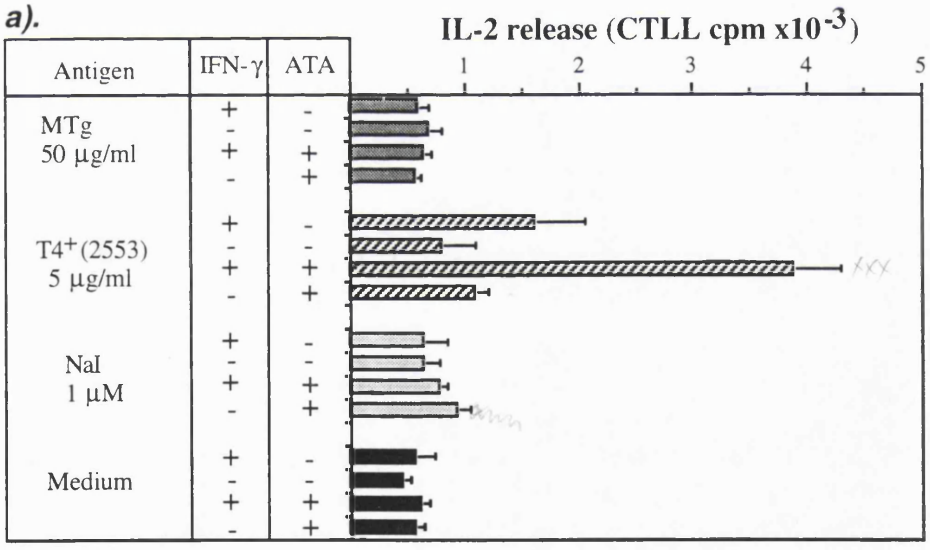
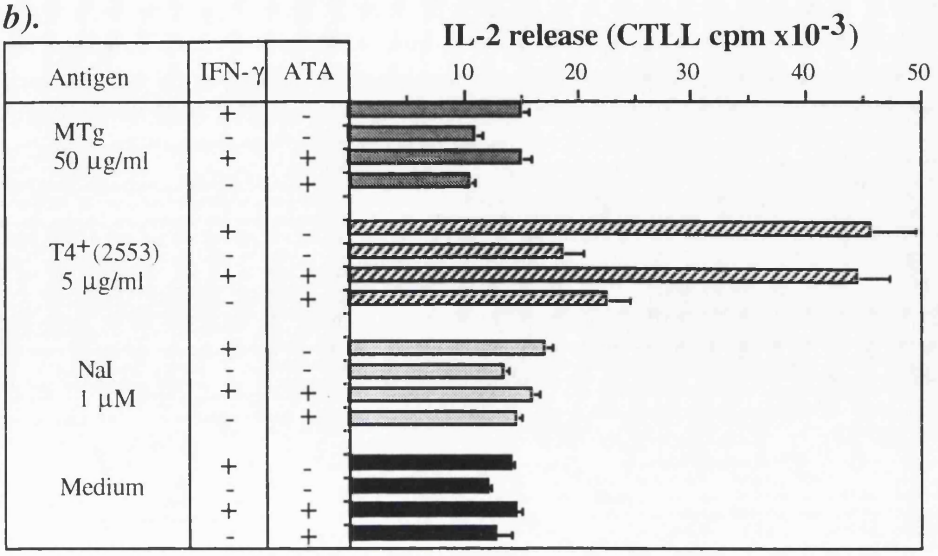


Figure:- 5.2. The presence of ATA does not consistently improve thyroid antigen recognition.

Response of $2x10^5$ CH9 to antigenic presentation by 5 day CBA/Ca thyroid epithelial cell cultures in the presence or absence of 1% ATA in the culture medium from day 1, as indicated in the figure, the presence or absence of IFN-γ is also indicated. Responses to 50μg MTg (-▩-), 5 μg $T4^+$(2553) peptide (-▨-), 1μM NaI (-□-) and medium control (-■-) are shown. Panels ***a)*** and ***b)*** (shown on the next page) show representative repeat experiments and illustrate the effect of ATA on the presentation assay. Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM. Figure 5.2*b* is shown on the next page. (Non-significant values not indicated *p<0.05, **p<0.01 and ***p<0.001). See Appendix 3

b).


IL-2 release (CTLL cpm x10-3)
10
20
30
40
50
Antigen
IFN-γ
ATA
MTg
50 µg/ml
T4+(2553)
5 µg/ml
NaI
1 µM
Medium

5.3 The presence of TSH does not improve thyroid antigen recognition.

The absence of presentation of exogenously derived Tg may have been due to reduced endocytosis from the tissue culture medium; therefore following monolayer preparation the cells were incubated from day two in culture medium that had been supplemented by 0.1 µg/ml TSH, with a view to stimulating uptake of Tg from the surrounding medium. Figure 5.3 shows CBA/Ca derived thyrocytes presenting to CH9 in the presence or absence of TSH. It can be seen that TSH does not have any significant effect on the processing and subsequent presentation of Tg derived peptide, normal spleen cell controls for this experiment show recognition of Tg by CH9. There is no increase in the presentation of $T4^+$(2553) peptide to CH9, but there may be a small, but significant, increase in endogenously derived antigen presentation in the presence of TSH. However this level of stimulation is probably not biologically significant. The presentation of exogenously derived MTg was also not improved by the addition of up to 500µg/ml MTg prior to assay of presentation to CH9; TSH was also unable to improve this presentation (data not shown). These levels in culture are high enough to be comparable to that which would be obtained in the thyroid follicle in *vivo.*

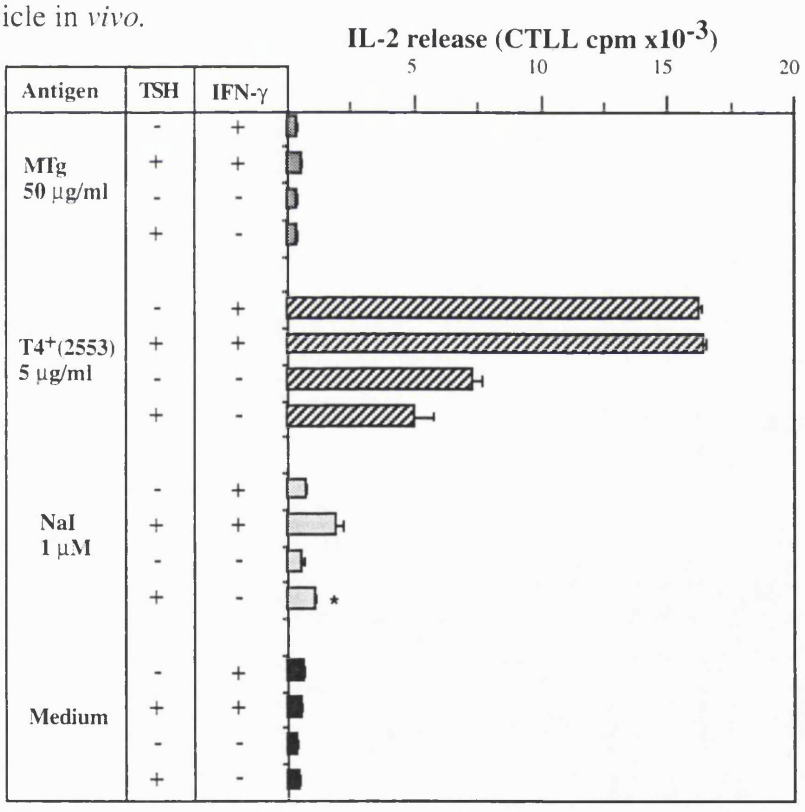


Figure:- 5.3. The presence of TSH does not improve thyroid antigen recognition.

Response of $2x10^5$ CH9 to antigenic presentation by 5 day CBA/Ca thyroid epithelial cell cultures in the presence or absence of 0.1µg/ml TSH in the culture medium from day 1, as indicated in the figure, the presence or absence of IFN-γ is also indicated. Responses to 50µg MTg (-□-), 5 µg $T4^+$(2553) peptide (-▨-), 1µM NaI (-□-) and medium control (-■-) are shown. Supernatants taken after 24h., for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM. (Non significant values not indicated * $p<0.05$, ** $p<0.01$ and ***$p<0.001$). See Appendix 4.

5.4 Thyroid epithelial cells cannot present antigen to primed lymph node cells.

Given the successful presentation of $T4^+$(2553) peptide to CH9 T-cell hybridomas, it might be expected that thyroid epithelial cells would be able to present antigen to MTg or $T4^+$(2553) peptide primed LNC. This might suggest an involvement of TEC in the perpetuation of thyroid disease as has been speculated given the expression of class II by human TEC in disease conditions. However figure 5.4 panels ***a)*** & ***b)*** show that no presentation to high responder (CBA/J) primed LNC was observed by syngeneic TEC's which had been previously pulsed with antigen. The LNC controls, ***c)***, to which the corresponding antigens were added gave positive proliferative responses to these antigen. MTg immunised LNC were clearly capable of responding to MTg and $T4^+$(2553) peptide and $T4^+$(2553) peptide immunised LNC were able to respond to the same peptide *in vitro.* Therefore, given that this protocol has previously been shown to allow presentation of the same antigens to CH9 T-cell hybridomas, it can be concluded that the thyroid epithelial cells are incapable of presenting these antigens to the primed LNC.

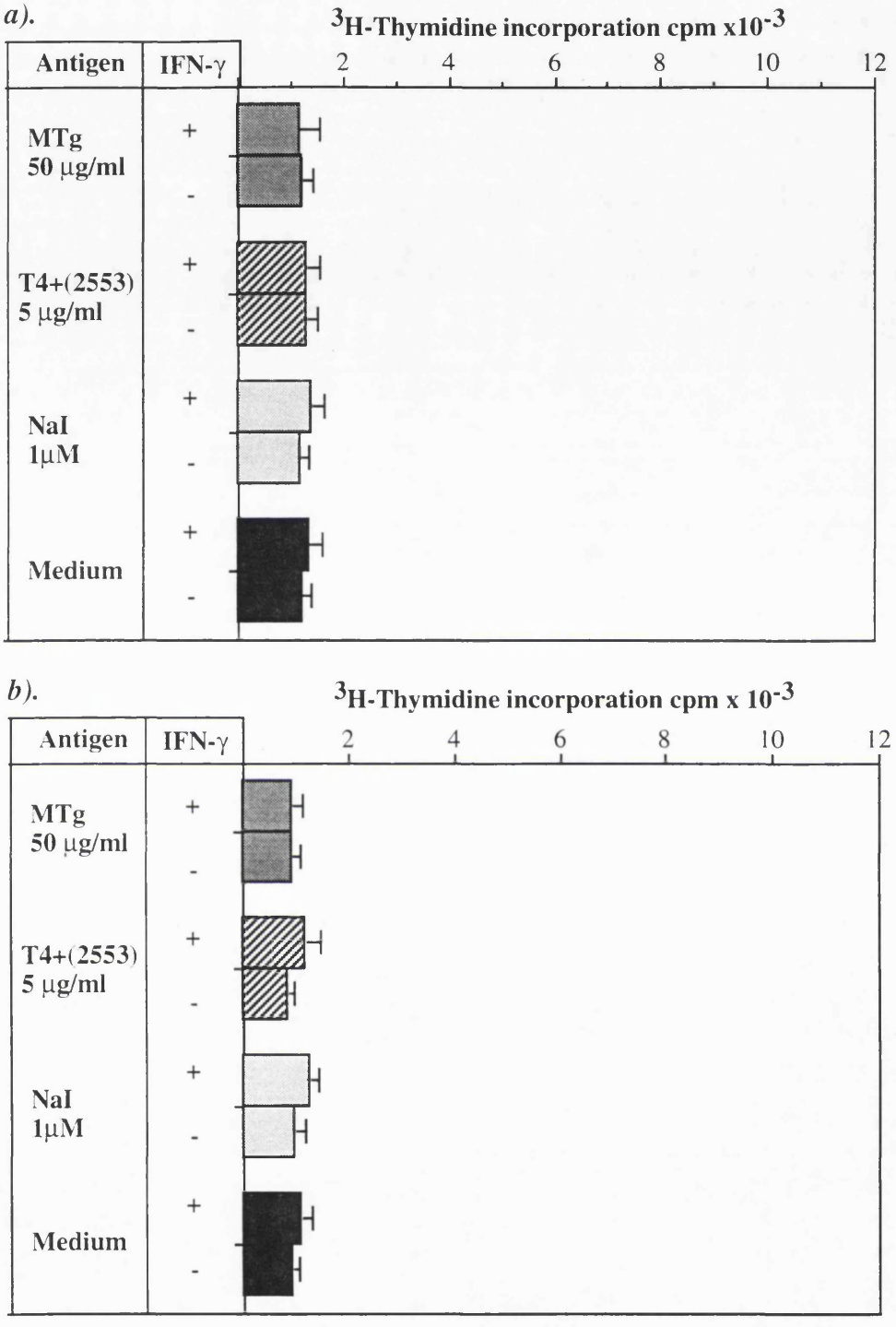
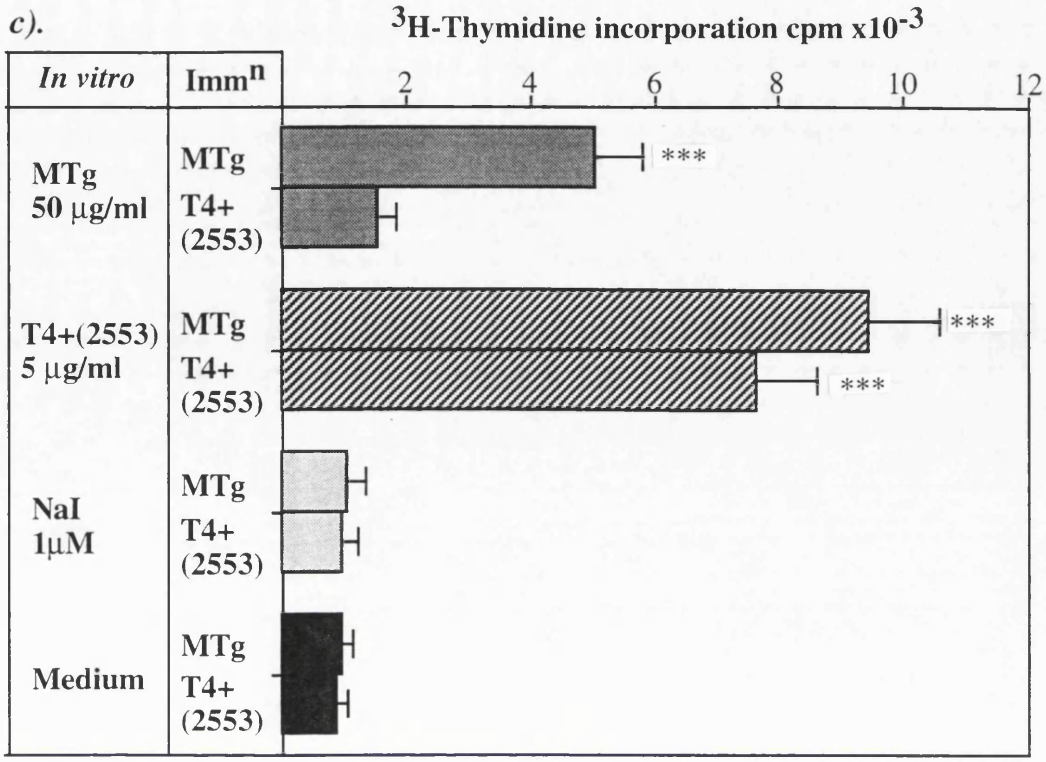


Figure:- 5.4. Thyroid epithelial cells cannot present antigen to primed lymph node cells.

Response of $2x10^5$ Day 16 LNC immunised with ***a)*** 50μg MTg/CFA or ***b)*** 5μg of $T4^+$(2553) peptide to antigenic presentation by 5 day CBA/J thyroid epithelial cell cultures in the presence or absence of IFN-γ as indicated in the figure. Responses to 50μg MTg (-□-), 5 μg $T4^+$(2553) peptide (-▨-), 1μM NaI (-□-) and medium control (-■-) are shown. LNC controls ***c)*** are shown on the next page. Proliferations were assessed by (^{3}H)-thymidine incorporation and results shown are average of triplicate cultures +/-SEM. (Non significant values not indicated *p<0.05, **p<0.01 and ***p<0.001). See Appendix 5.



5.5 Interferon-γ induces class II expression on thyrocytes and this is up-regulated by TNF-α.

The thyroid epithelial cells in the presence of IFN-γ clearly are able to present exogenously derived peptides to hybridoma cells but appear to be unable to present peptide to Tg specific lymph node derived T-cells in *in vitro* culture. Given the disease situation where activated T-cells home specifically to the thyroid gland there must be some form of presentation of a very specific thyroid antigen to enable the cells to home to the gland. Thus it may be postulated that in thyroid disease there are other factors which are important in thyroid antigen recognition which are absent in *in vitro* culture. In ongoing disease there are many cytokines which are locally present; thyroid infiltrating lymphocytes have been shown to produce IFN-γ and TNF-α locally thus there is a source of cytokine which is capable of inducing class II expression as has already been documented; it has also been shown that TNF-α receptors are expressed following treatment with IFN-γ which then renders cells susceptible to TNF-α (Tsujimoto *et al.*, 1986 & Ruggiero *et al.*, 1986). This is able to act in synergy with IFN-γ to further up-regulate class II expression (Weetman *et al.*, 1988). This is demonstrated on CBA/Ca thyroid epithelial cells. Figure 5.5 in which thyroid cultures were pre-incubated with IFN-γ for 48 h prior to the addition of TNF-α for 24 h, cells were then incubated overnight in Ca^{2+}/Mg^{2+} free HBSS to detach the cells and stained according to method 2.2.7I. Cells were then observed by fluorescence microscopy for the expression of class II on their surface.

Two population of cells were observed reflecting two apparent levels of class II expression, Some cells appeared very brightly stained (bright cells), whilst others although clearly positive has a reduced level of expression (dull cells). In the absence of any cytokine there

was a very small level of "dull" positively stained cells; this may be the reason for low levels of peptide presentation in some of the TEC presentation assays in the absence of IFN-γ. On the addition of IFN-γ at 100 U/ml the level of positively stained cells rises to approximately 40%, of this figure 5% are brightly stained cells. With the subsequent addition of increasing amounts of TNF-α from 20 - 5000 U/ml, the % of cells positive for class II rises from 40 % at 20 U/ml TNF-α to 75% of cells positive for class II at 5000 U/ml. This is accompanied by a steady rise in the % of the positive cells that are classified as bright rather than dull. This figure initially does not differ from that obtained with 100 U/ml of IFN-γ *i.e.* the % of cells staining brightly remains at 5% following the addition of 20U/ml of TNF-α. However, once the levels are increased to 100U/ml and above, there appears to be a dose response in the % of cells appearing to be brightly rather than dully stained and the % of brightly stained cells reaches 45%. Thus it is feasible that high local levels of these cytokines could lead to massive up-regulation of class II expression in the gland, therefore perpetuating the disease.

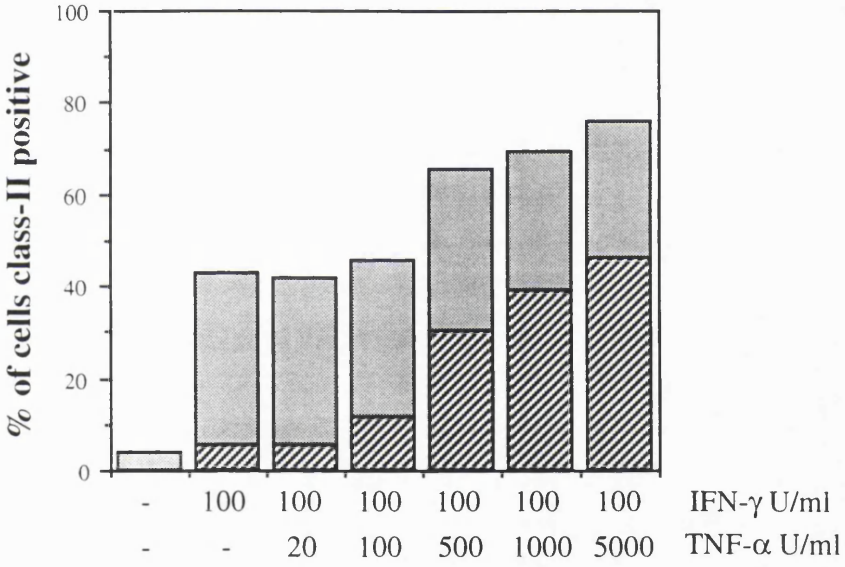


Figure:- 5.5. Interferon-γ induces class II expression on thyrocytes and this is up-regulated by tumour necrosis factor-α.

Thyroid epithelial cells from CBA/Ca mice were cultured in duplicate for 5 days. Following adherence after 24 h, cells were cultured with IFN-γ at the doses indicated for 24 h prior to the addition of TNF-α for the remainder of the 5 day culture period Cells were stained +/- MRC OX-6 rat anti-mouse class II antibody reactive to mouse H-2A^k followed by +/- FITC conjugated anti-rat Ig. Cells were mounted under coverslips in mounting medium for fluorescent microscopy and sealed. Duplicate cultures were assessed for number of cells in view and the number of cells class II positive or negative. Levels of background staining were assessed using cells stained with FITC conjugate alone and were shown to be < 0.5% of the total cells. Results of 'dull' (-▨-) or 'bright' (-▧-) by cells fluorescence microscopy are expressed as a % of the total number of cells observed under phase contrast and are an average of duplicate cultures.

5.6 High and low responder strains show no significant variation in the levels of class II induced by IFN-γ and TNF-α.

Given the differences previously indicated between the CBA/Ca and the CBA/J strain mice regarding the differing susceptibility to EAT induction even though they are both the same susceptible haplotype, it was investigated whether the CBA/Ca strain was more resistant to induction of class II antigens. Thyroid epithelial cells from each strain were cultured in the presence of IFN-γ with increasing amounts of TNF-α and the levels of class II staining on a single cell suspension was assessed as described previously. Figure 5.6 ***a)*** again shows that the level of total positive cells in the CBA/Ca strain was approximately 75%, with the number of brightly stained cells reaching 45%. In the CBA/J ***b)*** the increase in total positive cells and the number of brightly stained cells is dose dependent, the total number of positive cells reaching 90% and the number of bright cells, reaches approximately 50%. Although there is a slight increase on the number of cells staining positive and the percentage of bright cells, given the significant levels of up-regulation already seen in the CBA/Ca strain it is difficult to see that this small increase could account for the difference in susceptibility between the two strains. Figure 5.6 is shown on the next page.

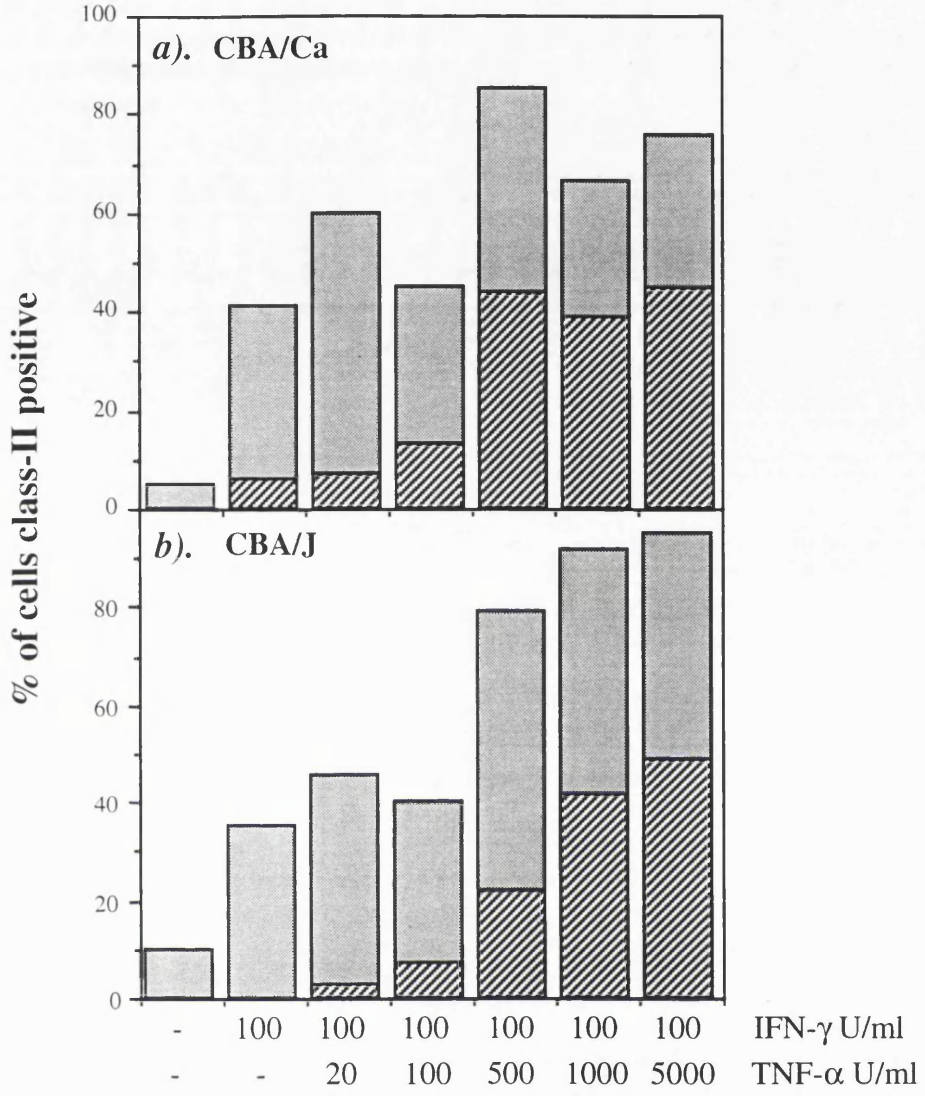


Figure:- 5.6. High and low responder strains of mice show no significant variation in the levels of class II expression induced by IFN-γ and TNF-α.

Thyroid epithelial cells from CBA/Ca (panel ***a)***) and CBA/J (panel ***b)***) mice were cultured in duplicate for five days. Following adherence after 24 h, cells were cultured with IFN-γ at the doses indicated for 24 h prior to the addition of TNF-α for the remainder of the five day culture period Cells were stained +/- MRC OX-6 rat anti-mouse class II antibody reactive to mouse H-2A^k followed by +/- FITC conjugated anti-rat Ig. Cells were mounted under coverslips in mounting medium for fluorescent microscopy and sealed. Duplicate cultures were assessed for number of cells in view and the number of cells class II positive or negative. Levels of background staining were assessed using cells stained with FITC conjugate alone and were shown to be < 0.5% of the total cells. Results of 'dull' (-▩-) or 'bright' (-▨-) by cells fluorescence microscopy are expressed as a % of the total number of cells observed under phase contrast and are an average of duplicate cultures.

5.7 TNF-α synergises with IFN-γ to up-regulate the level of antigen presentation by thyrocytes.

Does this up-regulation in class II by IFN-γ and TNF-α lead to a corresponding increase in the level of antigen presentation by thyroid epithelial cells? Figure 5.7 shows the effect of IFN-γ and TNF-α on the presentation of a fixed concentration of $T4^{+}$(2553) peptide (5 μg/ml) to ADA2. This data shows a number of things; firstly that the ADA2 hybridoma recognises and responds to peptide presented in the same way as CH9. Therefore, although they appear to see the peptide slightly differently, two separately derived clones and their hybridomas can recognise antigen presented by thyroid epithelial cells. Secondly, TNF-α is able to act synergistically with IFN-γ, not only to up-regulate the expression of class II but to up-regulate the presentation capacity of thyroid epithelial cells to specific T-cells; as seen in both panels ***a)*** and ***b)*** from figure 5.7. Panel ***a)*** shows the response of CBA/Ca thyroid epithelial cells to a dose response curve of TNF-α in the presence or absence of IFN-γ. It can be seen that without IFN-γ to induce TNF-α receptor expression, there is no presentation of peptide to ADA2. However, if IFN-γ is added at a level of 100U/ml in addition to a dose response curve of TNF-α, there is a dose dependent increase in the level of stimulation up to 1000U/ml of TNF-α. Above 1000U/ml of TNF-α there is a total loss of effect which is probably due to the cytotoxic effects of TNF-α at high concentrations. This pattern is mirrored in panel ***b)*** which shows the effect of the same set of conditions on CBA/J thyroid epithelial cells. Again there is no response in the absence of IFN-γ. On the addition of IFN-γ and a dose response curve of TNF-α, there is a concomitant increase in the level of presentation to ADA2; however it appears that the CBA/J may be slightly more sensitive to the cytotoxic effects of TNF-α than the CBA/Ca. There is a drop in the level of presentation at concentrations of TNF-α between 100 and 1000 U/ml and at 5000 U/ml the response is at background levels. Figure 5.7 is shown on the next page.

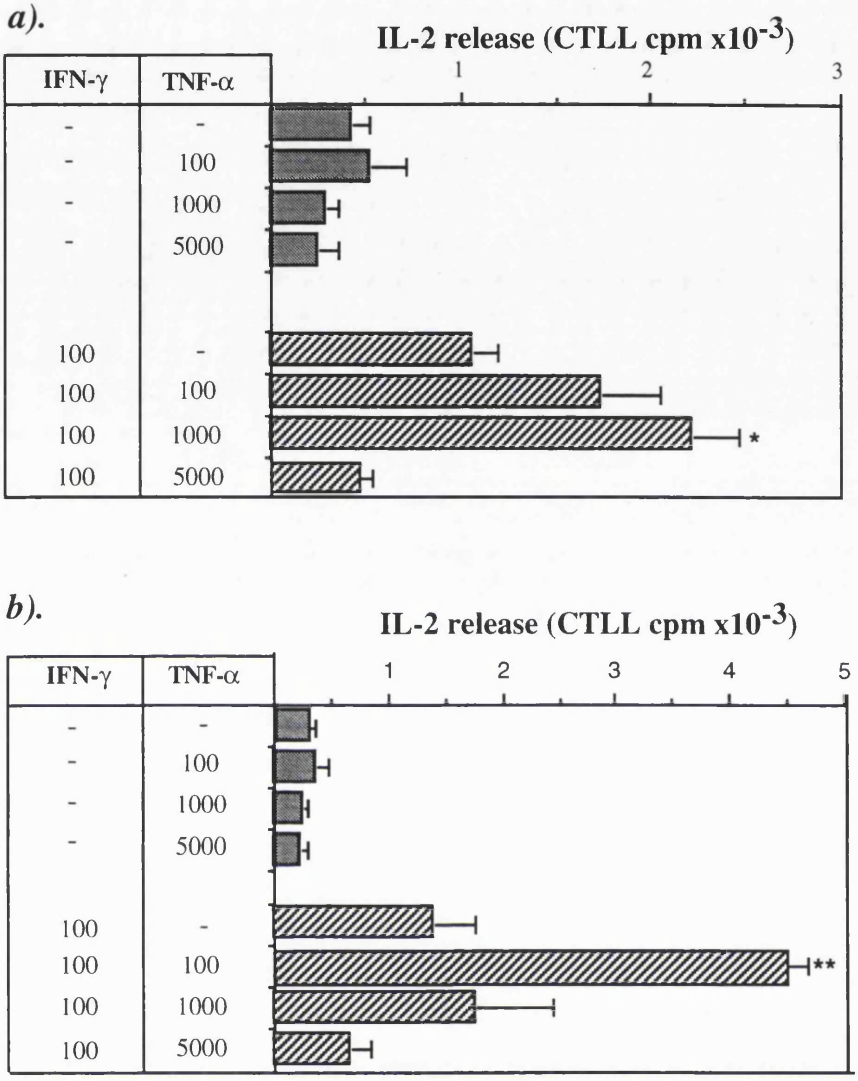


Figure:- 5.7. TNF-α syngergies with IFN-γ to up-regulate the level of antigen presentation by thyroid epithelial cells.

Response of $2x10^5$ ADA2 to antigenic presentation by five day thyroid epithelial cell cultures. IFN-γ was added the culture medium for 24 h prior to the addition of TNF-α for the remainder of the culture period. For the final 18 h of the culture period 5 μg/ml $T4^+$(2553) peptide was added prior to addition of CH9. Responses of CH9 to presentation of $T4^+$(2553) peptide by CBA/Ca TEC are shown in ***a)*** and responses to presentation by CBA/J TEC are shown in ***b)***. The level of presentation observed due to the presence of TNF-α alone is shown in (-▩-) and the level seen in the presence of TNF-α and IFN-γ is shown in (-▨-). Supernatants taken after 24h, for assessment of IL-2 release, results shown are average of triplicate cultures +/- SEM. (Non significant values not indicated * $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

6.0. Discussion.

Analysis of the *in vitro* and *in vivo* T-cell epitope.

Iodination is important in the induction of autoimmune thyroid disease in the OS chicken (Bagchi *et al.*, 1985) and BB rat (Allen *et al.*, 1987) and appears to heighten the severity of thyroiditis in the human. The thrust of the present thesis is to support the hypothesis that the major effect of iodine is to boost the role of hormonogenic sites in stimulating pathogenic T-cell clones responsible for the infiltration and attack on the thyroid gland.

Previous work by Champion *et al.*, (1987) demonstrated that iodination levels of thyroglobulin are important for the induction of EAT in the susceptible strain mouse CBA/J (I-A^k). Non-iodinated (ATA) Tg although unable to induce lymphocytic infiltration of the thyroid, was able to induce the production of autoantibody at a comparable level to that induced by fully iodinated thyroglobulin. Polyclonal antibodies produced in response to fully iodinated Tg and monoclonal antibodies to Tg were able to bind both normal and ATA-Tg equally well, suggesting that it is not necessary to have high levels of iodination of Tg for the presence of at least the majority of antibody determinants although some antibodies are against thyroxine itself. Epitope mapping of human thyroglobulin has shown antibody reactivities against the C-terminal (Dong *et al.*, 1989) and also against the central portion of Tg (Henry *et al.*, 1990), other workers have shown that autoantibodies can be generated against the hormonogenic sites (Den Hartog *et al.*, 1990). Clearly autoantibodies are inducible by various portions of the Tg molecule but are not restricted by the presence or absence of iodinated epitopes. The weight of evidence suggests that autoantibodies are not pathogenic in the human; the mediator of pathogenesis is the T-cell. This is supported by the data that shows that $CD4^+$ T-cells are absolutely required for the induction and transfer of disease in susceptible strain mice (Kong *et al.*, 1989b, Hutchings *et al.*, 1993) and that the $CD4^+$ parental clones of the hybridomas used in this study are capable of inducing disease in syngeneic mice(Champion unpublished results). It is clear that in the mouse H-2^k haplotype; ι iodinated epitopes are important in the pathogenesis of the disease

Champion *et al.*, (1985) reported the establishment of T-cell lines derived from H-2^k CBA/Ca mice, which were specific for mouse Tg and which were cross-reactive for rat, pig and human Tg. These lines were shown to be H-$2A^k$ restricted and $CD4^+$ (Champion *et al.*, 1986); from these lines two were selected for fusion with BW5147 fusion partner to create two T-cell hybridomas CH9 (derived from MTg12B) and ADA2 (derived from MTg9B3) (Rayner *et al.*, 1987). Both hybridomas were specific for syngeneic and allogeneic Tg they were also cross reactive with rat and human Tg; however both clones were shown to be distinctive by their TCR β chain rearrangements; thus although they were functionally similar they were clonotypically distinct from each other. Using preparations of human Tg which differed in their iodine content, poorly iodinated Tg was not able to stimulate

either of these two T-cell hybridomas. On increasing the iodination, the levels of stimulation also increased (Champion *et al.*, 1987); thus the cells recognise Tg only if it is sufficently iodinated. This implies that the hybridomas are recognising antigen which is related to the hormonogenic sites. Using tryptic digests of Tg in which the resultant peptides were purified on an affinity column to produce T4 depleted ($T4^-$) and T4 enriched ($T4^+$) pools; Champion *et al.*, (1991) were able to show that the reactivity of these two T-cell hybridomas was confined to the $T4^+$ enriched preparation; with no reactivity in the $T4^-$ preparation, this was further evidence that the reactivity of these cells was confined to the hormonogenic sites.

Following the publication of the structure of human Tg (Malthiery & Lissitzky 1987) we were able to synthesise overlapping peptides covering these sites, utilising the cross reactivity of these hybridomas to human Tg. Figure 3.1 shows that one of these T-cell hybridomas; CH9 is not only able to recognise iodinated determinants but that this reactivity is confined to a particular hormonogenic site namely residue 2553 on human Tg. It has also been shown that ADA2 is also able to recognise this epitope although there may be a slightly different pattern of recognition due to the different TCR used (Champion *et al.*, 1992). This is the first direct evidence that Tg autoreactive T-cells are capable of the recognition of iodinated residues which has important implications in the pathogenesis of autoimmune thyroid disease in the human given the implicated role of iodine in the development of the disease.

In the iodination reaction of Tg involving TPO; the first residue to be iodinated is at position 5 followed by the other residues. At residue 5 the T4 iodothyronine is preferentially formed; this is in contrast to the C-terminal residue 2746 which is believed to preferentially form T3. The central residues, 2553 and 2567 which lie very close to each other are probably T4 residues given the ratio of T4 and T3 found in normally iodinated Tg.

Lamas *et al.*, (1989) have shown that of the 67 tyrosine residues in the Tg molecule, 16 can be iodinated, three residues 5, 2553 and 685 are able to provide the inner iodothyronyl ring for further iodination and can be iodinated early. Hormonogenesis requires the initial iodination of two tyrosine residues which are the acceptor and donor tyrosines, their subsequent coupling leaves iodothyronine at the acceptor and dehydroalanine at the donor sites (Gavaret *et al.*, 1980); therefore there seems to be some evidence that residue 2553 could be preferentially iodinated as well as residue 5.

The C-terminal portion of Tg has been shown to be most susceptible to proteolysis in the serum (Marriq *et al.*, 1983) and it is believed that this residue is responsible for most of the T4 in the serum. However, it cannot be excluded that residue 5, because of its terminal position in the molecule may also be highly susceptible to proteolysis in the human. Therefore the two residues at the N and C terminals may be those most commonly liberated

in the serum following the release of thyroglobulin from the thyroid into the bloodstream. It is not known if proteolysis cleaves the T4 residue such that it forms part of a peptide before final cleavage resulting in the T4 hormone itself. If a peptide is formed as part of the natural degradation of the Tg molecule this has the potential for presentation by antigen presenting cells and subsequent recognition by T-cells.

Foetal thyroid function develops autonomously of the maternal thyroid system. The placenta is impermeable to TSH and only allows small amounts of T4 and T3 to cross; also the placenta contains a monodeiodinase that converts T4 to rT3 (reverse T3) and T3 to T2 which probably aids the low permeability of the barrier (Roti *et al.*, 1983). The foetal hypothalamic-pituitary complex is capable of synthesising TSH and TRH (thyroid releasing hormone) by 11-12 weeks. (Fisher and Klein 1981); TSH and T4 levels remain low until mid gestation and thereafter increase until term, T3 levels remain consistently below those of T4. In the newborn, the thyroid hormones are important in somatic and bone growth also dental and brain development. As the child develops into puberty the role of the hormones is to regulate hypothalamic and pituitary function; in the adult they are more involved in the regulation of metabolism as has been described in the symptoms of Hashimoto's thyroiditis and Graves' disease. Therefore at the time when the neonate is developing an immune system; thyroglobulin and it's breakdown products would be present in the circulation. Peptides produced as a result of Tg degredation would be able to reach the thymus and therefore should be subject to the normal mechanisms of central tolerance in the thymus. These autoantigens which are available to the circulation would not be sequestered and therefore should be presented in the thymus in sufficient levels to allow thymic deletion of autoreactive T-cells.

A problem in this theory arises if not all of the potential antigens on thyroglobulin become available to the immune system in ontogeny; a lack of sufficient concentration of antigen may allow some reactive T-cells to escape deletion. Alternatively, the iodinated epitopes may not bind with a great enough affinity to allow deletion; T-cells may be deleted which are reactive against immunodominant epitopes and the iodinated epitope reactive T-cells may escape deletion as they are reactive against sub-dominant epitopes. It is only on release to the periphery that these T-cells then have the potential to be autoreactive if serum antigen levels are high enough or that because of a lack of immunodominant antigen reactive T-cells, the population of T-cells responding to sub-dominant epitopes is allowed to expand. Given the preferential proteolysis of certain parts of the Tg molecule it is possible that certain epitopes on the Tg molecule may be released in preference to other epitopes; this may result in the tolerance to certain hormonogenic regions such as at residues 5 and 2746 and lack of tolerance to regions at 2553 and 2567 which are in the central portion of the molecule.

It is perhaps no surprise that the all portions of the molecule seem to contain B-cell determinants, given that antibodies recognise conformational determinants and therefore

do not rely on the breakdown of the molecule. T-cells recognise linear determinants and thus are dependent on the breakdown of Tg prior to recognition. Although proteolytic enzymes will cleave in defined sites, this cleavage may be affected by levels of glycosylation or the production of these enzymes may vary in an individual. These may in turn be affected by the state of the individual; whether they are stressed, suffering an infection or have already suffered thyroidal trauma resulting in damage. Thyroidal trauma may result in high levels of thyroglobulin bring released which allows the levels of normally sequestered epitopes in the periphery to be raised. Either effect would allow the production of iodinated epitopes that may not have been presented in thymic ontogeny resulting in the individual not being tolerant.

It is clear that Tg autoreactive T-cells can recognise peptides which contain T4 residues, in particular the H-2A^k restricted mouse is able to respond to peptide covering the residue at position 2553. Therefore this residue is effectively presented by H-2A^k restricted class II to syngeneic T-cells. The crystal structures for human class I and class II have now been determined (Jardetzky *et al.*, 1991 & Stern *et al.*, 1994) which allow us to see the types of peptides that are able to bind. The class I molecule consists of a β-pleated sheet upon which two anti-parallel strands form a closed cleft in which peptide is able to bind. As the cleft is closed, only peptides of about 9aa are able to fit into the groove; these peptides are anchored into the groove by their N and C termini. The peptides are further anchored by hydrogen bonding with the MHC and depending on the MHC type, by particular allele specific anchor residues within the peptide which bind down into binding pockets in the molecule. Longer peptides are accommodated by the bulging of peptides out of the groove. This appears to define the specificity of the MHC for particular families of peptides that are able to fit and have anchor residues that are able to make the correct connections within the MHC cleft.

A similar situation applies to the class II MHC except that the ends of the groove are open which allow the fit of a longer peptide which is anchored by about five allele specific binding residues along its length. The peptides that are associated with class II are longer than those found bound to class I and are believed to be of greater than 15aa residues in length, allowing their termini to trail out of the class II cleft. The minimal epitope that we have defined that is involved in binding and recognition of H-2A^k restricted cells is 9aa and the optimal size of the peptide that activates Tg specific hybridomas is 11aa. We know that this peptide associates with H-2A^k as the response to this peptide is inhibited by anti-class II antibody in a dose dependent manner; this is in common with the anti-class II inhibition of the response to native Tg. This peptide is smaller than usually associated with class II molecules; however given that the class II cleft is open there would appear to be the possibility of a degree of variation in the length of peptides that may associate or that longer peptides may be trimmed leaving shorter peptides. This may account for our observation of a minimal peptide of only 9aa. It is interesting to note that the peptide that

we have identified as binding to H-2A^k has a very defined structure C-terminal to the thyroxine residue. Additional residues beyond lysine 2561 serve to diminish the peptide potency indicating that the C-terminal portion positioning in the groove of class II in relation to the thyroxine may be critical to recognition; this may be the location of class II or TCR binding residues which are important in the positioning of the peptide. The data suggests that this portion of the peptide may be firmly fixed in the class II cleft; whereas the N-terminal portion of the peptide may be directed towards an open end. This may be substantiated by the observation that addition of residues N-terminal to the 11aa peptide from serine 2549 seem to have little effect on the overall peptide reactivity; these residues may extend out of the class II molecule and be clipped.

Clearly particular amino acid residues play an important role in the recognition of the peptide; we also know that the iodination of Tg is also important in the recognition of Tg by the autoreactive T-cell hybridomas used in this study. This implicates the thyroxine residue and in particular the iodination state itself in the recognition by T-cells. We have been able to show that an iodinated thyroxine residue is essential to the recognition of this peptide by our Tg reactive T-cell hybridomas. This recognition is highly specific as none of the naturally occurring amino acids are able to substitute for the reactivity of thyroxine even an acceptor tyrosine residue; this implicates either the double benzene ring structure of thyroxine or the iodination state in peptide reactivity. Further to this we have shown that this 11aa peptide, where the T4 residue is substituted by a benzyloxyl residue which has the same structure of T4 but is not iodinated; is totally non-stimulatory to both T-cell hybridomas. This strongly implicates the iodine atoms in the recognition of the peptide by the T-cells, however the iodinated ring structure alone is insufficient to stimulate the T-cell hybridomas as free T4 is also non-stimulatory. Therefore the iodinated T4 residue has to be seen in the context of a peptide backbone for recognition; which is not surprising given the structure of class II.

The T4$^+$ (2553) peptide has now been shown to be capable of causing thyroiditis (Hutchings *et al.*, 1992), however at this present time we have been unable to induce thyroiditis *in situ* but have been able to use a transfer protocol. Lymph nodes from mice that have been immunised with whole Tg or T4$^+$(2553) peptide, when cultured *in vitro* with this peptide are able to transfer thyroiditis. This *in vitro* step has been shown to be critical to the transfer of EAT in that *in vitro* activation with a pool of tryptic peptides lacking T4 are unable to stimulate cells *in vitro* and therefore transfer disease. This transfer of EAT using iodinated peptide is inhibitable with anti-CD4 monoclonal antibody as is the MTg transfer of disease indicating that the CD4$^+$ T-cell pool is a critical responding population.

Given that the benzyloxyl and T4$^-$ peptides are non-stimulatory, it might be expected that these peptides would be able to block the recognition of the iodinated T4 containing peptide

as they have the same peptide backbone. However, it was impossible to demonstrate any blocking ability of these peptides whether APC's were pre-pulsed or co-incubated with these peptides in the presence of stimulatory peptide and responding T-cells. This was surprising, they might have been expected to bind given the postulated requirement for the positioning of the T4 residue in relation to the C-terminal portion of the peptide. It is possible that it is not the amino acid backbone that is binding to the class II molecule but the T4 residue itself; although it is difficult to envisage how this structure would fit into the class II cleft. The residue is very large and accounts for 40% of the molecular weight of the peptide; it is also highly charged and this residue may be able to bind into the class II cleft.

However this theory does not fit in with the observation that anti-thyroxine antibody is able to block the recognition of APC's that have been preincubated with iodinated peptide; the addition of blocking antibody was able to bind thyroxine residues such that it prevented T-cell recognition of the peptide. This implies that the thyroxine residue might be exposed as the antibody is able to block following preincubation of iodinated peptide and APC's and therefore this suggests that the TCR may be recognising the iodinated T4 residue. As shown in previous data, iodination is critical for the T-cell recognition as the benzyloxyl containing peptide is non-stimulatory, this implies that iodine could be critical for binding to TCR. Unfortunately we were unable to test this peptide with T3, diiodothyronine and monoiodothyronine residues in the place of the T4 residue. It would have been interesting to see if the T-cell recognition is dependent on a certain level of iodination; for example is the level of stimulation proportional to the number of iodine atoms or is the pattern of iodination the critical factor. These two sets of data seem to imply that the T4 residue may be involved in TCR and MHC binding the diagram (Figure 6.1) below suggests how this might be achieved.

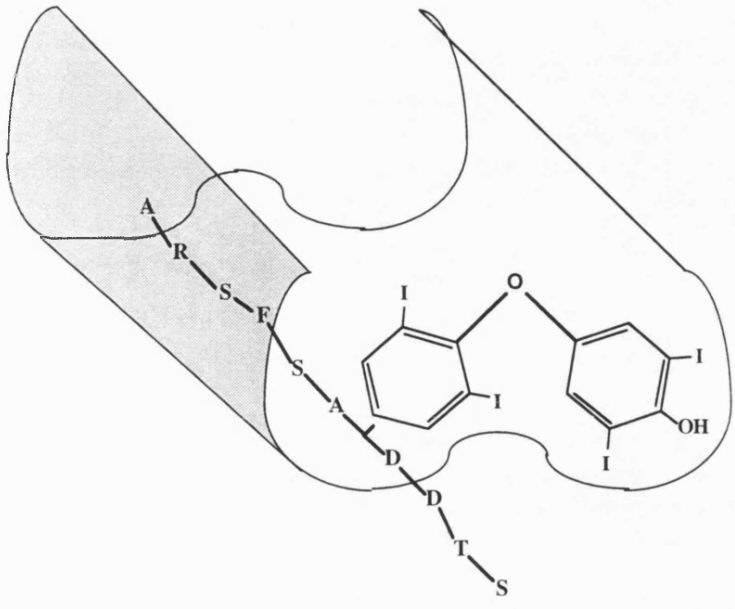


Figure 6.1. Schematic representation of T4*(2553) peptide in class II cleft (not to scale).

The TCR which has been shown to be employed in the recognition of this iodinated peptide by the MTg12B parental T-cell line to CH9 hybridoma is shown to be a Vβ8. How relevant this is to the human disease is questionable as the factors governing the TCR repertoire in the mouse such as Mls are not directly transferable to the human. Oligoclonality of TCR Vβ usage has been demonstrated in several experimentally induced autoimmune diseases (Burns *et al.,* 1989, Urban *et al.,* 1988, Gregerson *et al.,* 1991); use of the Vβ8 TCR has been demonstrated in thyroiditis in the NOD mouse (Matsuoka *et al.,* 1993). The MHC repertoire in the human is far more varied as the population is outbred whereas the experiments conducted in mice are on inbred populations which all have the same MHC. There is the potential that in the human population there would be a range of TCR's that would be able to recognise relevant peptides; it is unlikely that one TCR would be responsible for the development of the disease but there may be a restriction on the TCR's that are able to respond to thyroiditogenic epitopes. T-cell Vα and Vβ usage in Graves' disease and Hashimoto's thyroiditis has been shown to be multiclonal (Lipoldova *et al.,* 1989, McIntosh *et al.,* 1993), whereas other workers have demonstrated a restriction in the Vα usage (Davies *et al.,* 1991). I believe that it is highly likely, given the weak disease association with MHC and the evidence that environmental factors such as iodine in the diet and pathogens are believed to play a role in disease, that the conditions that lead to full development of disease may be highly varied and in the future be very difficult to predict.

The pathogenic peptide described in this thesis is not now the only one to have been identified as being involved in the pathogenesis of EAT. Salamero *et al.,* (1987) showed that a 5-10 kDa tryptic fragment from porcine Tg was able to induce thyroiditis *in situ*; this fragment would have contained a number of epitopes and the authors did not indicate which part of the Tg molecule this fragment covered. By 1992, Texier *et al.,* (1992b) were able to define the thyroiditogenic region from porcine Tg, publishing data that a 40aa peptide was able to induce EAT. Following sequencing of their original fragment they were able to align this with the published sequence of human Tg and synthesise the 40aa peptide. The peptide corresponds to positions 1672-1711 of human Tg containing one tyrosine but as it was synthetically made it is unlikely to be iodinated. The authors suggest that this sequence of 40aa contains a peptide that is recognised by Tc cells rather than Th cells; but do not give any indication whether their thyroid cytotoxic HTC2 hybridoma is capable of responding to this peptide.

A second group have demonstrated that a 17aa peptide covering residues 2495-2511 of human Tg is also thyroiditogenic (Chronopoulou and Carayanniotis 1992), but have used different mouse strains from ourselves and Texier *et al.,* (1992). They used the high responder mouse strains of SJL ($H\text{-}2^s$), C3H ($H\text{-}2^k$) and B10BR ($H\text{-}2^k$). The use of SJL may have some bearing on the fact that the peptide was different from our thyroiditogenic peptide; the other $H\text{-}2^k$ strains, although being the same MHC may process Tg differently

and therefore be capable of recognising epitopes other than that we have identified. This is supported by the fact that the peptide identified by Texier *et al.*, (1992b) is different but still pathogenic in CBA/J mice. Chronopoulou & Carayanniotis (1992) suggest that this peptide may be sub-dominant as LNC primed against MTg are unable to respond to this peptide; conversely, LNC primed to peptide are unable to respond to MTg. This is in contrast to our own results in which priming to whole molecule also primes to $T4^{+}$(2553) peptide. The authors also report that in common with our own observations they are unable to induce thyroiditis *in situ* with peptide 2495-2511 but can on *in vitro* activation and activated cell transfer.

This data correlates well with some observations made in figure 4.4, in which it is shown that other known susceptible strains other than CBA/J ($H\text{-}2^{k}$), such as CBA/Ca ($H\text{-}2^{k}$), DBA/1 ($H\text{-}2^{q}$) and SJL ($H\text{-}2^{s}$) have differing responses to peptides when immunised with MTg. As expected, the CBA/J and CBA/Ca both respond to peptides covering residue 2553 of human Tg; however the DBA/1 mouse seems to recognise iodinated epitopes, not from region 2553 but from region 5. In contrast, the SJL seems not to recognise any of the thyroiditogenic epitopes but still makes a good response to MTg. It may be that the epitope that the SJL is recognising is indeed that described by Chronopoulou and Carayanniotis (1992). We also have some data from D Rayner (unpublished) that our $T4^{+}$(2553) peptide is unable to induce thyroiditis in the DBA/1 and SJL when compared to the CBA/J and CBA/Ca. Taken together it would seem that the thyroiditogenic peptide that we have identified is not the only pathogenic epitope on Tg for the CBA/J mouse, but it may be an immunodominant Th epitope; this may account for our inability to induce EAT using iodine depleted Tg. Alternatively, the non-iodinated epitopes that have been identified in susceptible strains apart from the CBA/J may be cryptic determinants which are not necessarily involved in spontaneous thyroiditis. Given that this peptide and another thyroiditogenic peptide reported (Chronopoulou & Carayanniotis 1992) are unable to induce thyroiditis *in situ,* it may also suggest that other epitopes are necessary for the development of disease. The *in vitro* culture step will selectively expand the peptide specific population but may also via bystander help, expand other crucial populations which help in the development of pathogenesis. This may explain the ability of the 40aa peptide reported by Texier *et al.*, (1992b) to cause EAT *in situ*; this peptide is much longer than our peptide or that of Chronopoulou and Carayanniotis (1992) and therefore may contain greater than the one Tc epitope the authors predict. The *in vitro* culture step may also deplete the suppressor T-cell population allowing the outgrowth of pathogenic T-cells

Further to the recognition of the peptide by the Tg reactive hybridomas and the importance of the thyroxine residue, we have made attempts to delineate the role that the amino acid backbone plays in the recognition of peptide. Using 11aa peptides in which the residues except T4 were substituted with a limited range of amino acids we have been able to identify the residues that are critical to peptide stimulation of both the CH9 and ADA2 hybridomas.

From the results shown in figures 3.5 A and B it appears that the pattern of apparent residue importance in peptide recognition differs between the CH9 and ADA2. On first reflection this seems surprising as these hybridomas recognise peptide in the context of the same MHC, namely H-2A^k. However it has already been postulated that the T4 residue may be binding the MHC molecule; if this is the case, the difference in the residue importance between CH9 and ADA2 would reflect the different specificities of the TCR's. We do not know the affinity of the interaction between the TCR's of CH9 and ADA2 therefore it is entirely possible that the affinities of these TCR's are different which might account for the apparent distinct involvement of certain residues in activation of CH9 but a more generalised involvement of residues in the activation of ADA2.

It is clear that virtually any substitution of the peptide STDD(T4)ASFSRA at residues 8 (F=phenylalanine) and 10 (R=arginine) inhibits peptide responses by CH9 substantially. The only substitutions at position 8 that can retain peptide reactivity are R (arginine), N (asparagine) and K (lysine); R, N and K have relatively large side chains; these residues may be able to fulfill the function of the F residue as a result of their length or side chain size. It is interesting to note that Y (tyrosine) is unable to substitute for F, the only difference between these amino acid side chains is that Y has a hydroxyl group, this may affect the ability of this amino acid to substitute for F.

Of the eight amino acids used to substitute each residue, none are able to replace arginine at residue ten; this may indicate that this residue is the most critical one for peptide reactivity besides the thyroxine residue itself. Other residues display limited stimulatory capacity on substitution, residue 4, D (aspartic acid) cannot be substituted by G (glycine) probably because it is too small, the hydroxyl group of tyrosine again seems to affect its ability to substitute at this residue, this seems to also occur at residues 6,7 and 9. In general residues 1,2,3,4,6,7,9 and 11 can be substituted by a wide range of amino acids with little or no loss in peptide stimulatory capacity, residues 1,2 and 3 can be completely substituted with any amino acid tried. This is in keeping with the original observation that addition of residues N-terminal to the defined minimal epitope of 9aa have little effect on peptide reactivity; both sets of data suggest that this portion of the peptide is not involved in recognition by the TCR or MHC binding.

The sensitivity of the ADA2 hybridoma to substitution of the peptide is considerably less than seen for CH9. Residues 6 (alanine) and 7 (serine) seem to be the most affected by substitution and but not to the same degree as seen for CH9. It is difficult to see any pattern in the amino acids that are able to substitute for the alanine residue. Alanine has the smallest side chain of the amino acids therefore it might be expected that a small amino acid would make a successful substitution, this seems not to be the case in that the aromatic amino acids such as Y and F, and a large basic side chain such as R are able to substitute. If these substitutions were conferring on the peptide a greater ability to bind to MHC or TCR

it might be expected that these substitutions would have a stimulatory effect, they do not and it is difficult to envisage how these substitutions have so little effect when other substitutions of alanine for glutamic acid (E), lysine (K), leucine (L) and in particular glycine (G) have a negative effect. The effect is somewhat reversed at residue 7, where substitution of the serine by aromatic amino acids such as proline (P) and tyrosine (Y) and the long side chain amino acid arginine (R) affects peptide stimulation; the residue function seems to be little affected by substitution with the other amino acids. This would be more in keeping with the assumption that this is the or one of the residues involved in MHC or TCR binding. The only other residues that seem to be partially affected by any substitution are residues 10 where arginine is substituted by tyrosine and residue 11 where alanine is substituted by glutamic acid. In summary the overall level of activity of the residues is low for the ADA2 hybridoma, it is difficult to speculate why this is so. It may suggest that the peptide is not really affecting the recognition by the TCR, possibly as result of the T4 residue itself binding class II and TCR. This would be a highly unexpected occurrence given the current dogma regarding antigen presentation but given these and results previously mentioned it is impossible to rule out the possibility that T4 itself may bind class II, with the TCR of CH9 binding residues 8, 10 and T4 of the $T4^{+}$(2553) peptide.

It would have been interesting to look at a peptide which had the T4 residue at position 5, F at position 8 and R at position 10 with alanine spacer residues replacing the remaining residues and looking at the stimulatory capacity of the peptide. It might be expected that this peptide would be stimulatory to the CH9 hybridoma given the apparent lack of involvement of the remaining residues in stimulation. At this point we have been unable to delineate the residues that are responsible for the binding of the peptide to the MHC. The alanine spacer peptide (if stimulatory) or the original $T4^{+}$(2553) peptide could have been used to determine the affinity of the interaction with MHC. At the time of the cessation of this work; experiments had just begun to determine the affinity of the $T4^{+}$(2553) peptide for $H\text{-}2A^{k}$. Unfortunately these experiments were in their early stages and no results were obtained. However, given the affinity of a peptide for class II; it would have been possible to determine the MHC binding residues on the peptide by the substitution of likely residues such as 8, 10 and also T4 to see whether they affect the binding affinity of peptide in the presence of a detectable competitor peptide on H-2Ak positive cells. By a process of elimination we would be able to identify with some certainty the TCR binding residue/s.

The interaction of hen egg white lysozyme peptides with $H\text{-}2A^{k}$ have been studied by a number of groups. The immunodominant peptide of HEL is a 10mer peptide which has been shown to bind $H\text{-}2A^{k}$ (Allen *et al.*, 1987) and that this interaction could be stabilised by the addition of residues outside the minimal epitope (Nelson *et al.*, 1993). The addition of residues particularly at the N-terminal increased the stability of $H\text{-}2A^{k}$/peptide complexes and increased binding strength of peptide to class II, this is probably due to the contribution of contacting residues outside of the binding pockets or outside the peptide core. It is

suggested that the core comprises the antigenic determinant and flanking sequences increase stability of peptides for class II, this data may correlate with the data presented in figure 3.2. In this figure additions C-terminal to the core peptide slightly diminish peptide potency whilst addition N-terminal have little effect. However, the fact that N-terminal additions have little effect on the stimulatory capacity of the peptide when presented to T-cell hybridomas does not mean that those additions are not affecting the stability of the H-2A^k/peptide complex which may be important in the presentation of this peptide *in vivo* to autoreactive T-cells. However, given time constraints we were unable to measure the affinity of the interaction between different length peptides and H-2A^k.

The immunodominant peptide of HEL that binds to H-2A^k is a 10mer and has the sequence DYGILQINSR (Allen *et al.*, 1987), interestingly residues nine and ten of this peptide correspond to residues nine and ten of the T4$^+$(2553) peptide studied in this thesis. Peptide substitution experiments shown in figure 3.5A show that residue ten, arginine (R) is the residue that is least amenable to substitution by the amino acids used, suggesting that this residue may indeed be a critical residue for peptide reactivity in particular may be involved in binding to H-2A^k. A study by Altuvia *et al* (1994) has recently been published identifying common sequence motifs of antigenic peptides binding to H-2^k (I-A and IE). The motifs were predicted on a number of chemical and structural properties that may be involved in binding specificity such as hydrophobicity, charge, hydrogen bonding and size. Three sequence motifs have been identified for H-2A^k and the HEL motif described earlier fits motif A; this requires a hydrogen bond acceptor in the first position of the motif, a hydrophobic, aliphatic, average sizes amino acid in the fourth and an uncharged hydrophobic residue at the seventh. Our T4$^+$(2553) peptide fits the first two of these criteria in that the second aspartic acid (D position 2552) corresponds to position 1 on motif A and serine (S position 2555) corresponds to position four. However arginine (R position 2559) does not fit in with the motif as described. It is not hydrophobic rather it is hydrophillic and charged. The HEL peptide described by Allen *et al* (1987) also only has two of three residues that fit the motif and is stimulatory, however two of the residues identified as critical to peptide reactivity with CH9 T-cell hybridomas do not fit this motif. It is possible that when binding to H-2A^k, phenylalanine (2556) and arginine (2559) are TCR binding, possibly leaving T4 to bind MHC. Obviously this structure has not been documented as binding to I-A^k because it is not found in any other peptide studied to date. If phenylalanine and arginine are TCR binding this may account for the differences seen between CH9 and ADA2 in figures 3.2A and 3.2B.

It is interesting to note that the T4$^+$(2553) peptide also contains a motif for I-E^k binding. Motif C for I-E^k binding requires that position one is neutral (D position 2552), position 3 should be hydrophobic (A position 2554), position five should be neutral (F position 2556) and position seven should be hydrophillic and polar (R position 2557). The T4$^+$ peptide fits this motif perfectly and raises the possibility that although the T-cell hybridomas we

have been working on are H-2A^k restricted and present Tg and T4$^+$(2553) peptide, it may be that the pathogenic cells *in vivo* may be I-E^k restricted but still present the same peptide The CBA/Ca and CBA/J mouse both are H-2^k restricted with respect to I-A and IE, this raises the possibility that this peptide may be presented *in vivo* by both sets of class II (I-A and I-E) but in a slightly different form. The peptide may bind to I-A with phenylalanine and arginine mediating TCR binding and T4 mediating class II binding. The peptide may also bind I-E with the residues contributing to both TCR and class II binding resulting in a stronger interaction.

Antigen presentation by thyrocytes.

The role that the thyroid cell has to play in autoimmune thyroid disease is far from clear, following the observation that thyroid cells during disease in the human are able to express class II antigens, their role as potential initiators of or mediators of disease has been greatly investigated.

Great difficulty was encountered reproducing results achieved by a previous member of the department regarding the capability of TEC's to present MTg to the T-cell hybridomas. These hybridomas have a natural tendency to loose their responsiveness to MTg and had to be re-cloned a number of times to maintain responsiveness. In all of the thyroid cell experiments outlined in this thesis the specificities of these hybridomas to MTg and to $T4^{+}$(2553) peptide were checked using syngeneic spleen cells; appropriate negative controls such as $T4^{-}$(2553), peptide, NaI and medium controls were included in each experiment. CH9 and ADA2 in all of the experiments reported responded positively to MTg and $T4^{+}$(2553) peptide and to avoid repetition were not included in the results section. The medium that was used in previous experiments was supplemented with hormones but in my hands proved to be no more effective in the antigen presentation assay the basal medium used by Yeni and Charreire (1981); also the timing of the previously used assay (12d instead of 5d) did not enhance presentation of $T4^{+}$(2553) peptide or allow the presentation of MTg. Therefore, the simplified culture schedule was employed in all subsequent experiments as it seemed to allow the effective presentation of peptide although MTg presentation was not observed.

The addition of 1% ATA to the culture medium, to prevent the formation of iodinated Tg, was postulated might bring down the levels of background proliferations to allow the observation of proliferation of hybridomas in response to MTg presented by TEC's. This was unsuccessful and seemed not to affect levels of background proliferations. The addition of TSH was also unable to enhance presentation of MTg to CH9 hybridomas. It was postulated that the presence of TSH might enhance uptake of Tg from the culture medium into the thyroid cell, the addition of up to 500 µg/ml of MTg to the culture medium also failed to enhance presentation. It is possible that the thyroid cell may be unable to take up Tg whilst in *in vitro* culture. It is known that the polarity of the thyroid cell changes upon *in vitro* culture, this may substantially affect the capability to take up Tg by receptor mediated endocytosis; the cells may also loose their capacity to degrade Tg in culture. It was postulated that Tg produced endogenously by the thyroid cell, might be presented if the cell is supplied with excess NaI in culture to ensure the production of iodinated epitopes. Again there appears to be no presentation of endogenous Tg to the Tg reactive hybridomas.

It is clear that thyroid cells are capable of presenting antigen under a defined set of *in vitro* conditions, i.e. the antigen must be in the form of peptide which does not require any further

processing. The peptide would either occupy empty class II molecules, displace existing peptides from class II if in a sufficiently high enough concentration to compete with existing bound peptide or more probably be taken into the endosomal compartment and loaded onto class II. What is clear from these experiments is that thyroid epithelial cells in culture seem unable to take up and process whole thyroglobulin antigen even when provide at concentrations comparable to that found in the gland lumen. This would suggest that the process by which thyroid follicles are prepared and cultured effectively destroys the follicular architecture to such an extent that the follicle seem unable to take up whole antigen even when stimulated with TSH which should stimulate uptake. However, given the conclusion that thyroid cells *in vitro* seem not to be able to process and present peptides from whole antigen this does not preclude the possibility that in an *in vivo* situation the follicle cells are able to have an antigen presenting capacity. Whether the presenting cells are able to initiate immune responses or prolong ongoing responses will be alluded to later in the text.

We have been able to show that exogenously derived $T4^+$(2553) peptide is effectively presented by TEC's to T-cell hybridomas and that this level of presentation is increased in the presence of 100U/ml of IFN-γ. We have also been able to confirm that the presence of TNF-α is able to increase the level of class II expression on cultured mouse thyrocytes and that there seems to be little or no difference between the levels of expression induced on CBA/Ca and CBA/J mice. The CBA/J mouse is more susceptible to the induction of EAT and it was postulated that this may be due to an increased level of class II expression in the thyroid; this was clearly not the case. We were also unable to demonstrate any difference between the capacity of thyroid cells from these two strains to present $T4^+$(2553) peptide to T-cell hybridomas.

There is some suggestion that the CBA/J thyroid cells are more susceptible to the cytotoxic effects of TNF-α; this may imply that there is greater thyroidal damage in CBA/J thyroiditis which may serve to enhance ongoing disease. If the gland is more susceptible to the cytotoxic effects of locally produced T-cell cytokines it is possible to envisage how normally sequestered antigen from the thyroid may become available for presentation by local thyrocytes which are expressing class II antigens as a result of the same locally produced T-cell cytokines such as IFN-γ and TNF-α. However the T-cells that would be present in the gland would be already activated and therefore more readily able to respond to thyroid antigen presented by class II in the absence of any other costimulatory signals.

The expression of class II molecules on target organs associated with the development of autoimmune disease is well documented. Following the first discovery of class II expression on human autoimmune thyroid cells by Hanafusa (1983), the presence of class II molecules on pathological tissue specimens includes human retinal pigment epithelial cells (Chan *et al.*, 1986) and human synovial fibroblasts (Boots *et al.*, 1994). The induction of class II

expression has been demonstrated on the murine thyroid epithelial cell line M5 (Stein & Stadecker., 1987) and thyroid cells (Salamero *et al.*, 1985), rat thyroid cells (Weetman *et al.*, 1988) human islet cells (Pujol-Borrell *et al* 1987), rat cerebral endothelium (Linke & Male., 1994), human and rat retinal pigment epithelial cells (Liversidge *et al.*, 1988a, Liversidge *et al.*, 1988b) and human cultured rheumatoid synovial fibroblasts (Boots *et al.*, 1994). Further to this Boots *et al* (1994) have shown that cultured human synovial fibroblasts are able to process and present exogenously provided tetanus toxoid antigen to a cloned T-cell lines although the level of presentation was determined to be approximately 10x less than for a comparable number of PBMC's. Rheumatoid synoviocytes have been shown to express adhesion molecules (Morales-Ducret., *et al* 1992) but there is no description of B7 expression on these cells. In many ways the data support the findings in thyroid epithelial cells; that many cell types can be shown to express class II but doubt remains about the ability of those cells to initiate an immune response in naive T-cells that not only require peptide to be correctly presented but require a host of costimulatory signals for full activation.

In summary, class II expression is found on cells from sites that are targets for autoimmune processes and that certain cell types are able to process and also present exogenously derived antigen to cultured T-cell clones. This situation is not dissimilar to the presentation of $T4^{+}$(2553) peptide to T-cell hybridomas. However the data which suggests that epithelial cell types have the capacity to act as antigen presenting cells *in vitro* under a restricted set of conditions does not imply that these very same cells are able to initiate autoimmune conditions *in vivo* however they may be able to prolong activation once pathogenic processes are initiated.

It is interesting to note that these thyroid epithelial cells although capable of presenting $T4^+$(2553) peptide to hybridomas are unable to present this same antigen to MTg or $T4^+$(2553) primed lymph node cells. This is in contrast to results obtained by Remy *et al.*, (1986) & Yeni *et al.*, (1981). It is known that T-cell hybridomas have less stringent requirements for activation than T-cell lines and clones. This is because T-cells require certain costimulatory signals such as B7.1 and B7.2 for Th1 cells and IL-1 for Th2 cells; T-cell hybridomas are constitutively primed and do not require the generation of IL-2 message and IL-2 production for proliferation. These results are in keeping with those published by Ebner *et al.*, (1987). These authors found that thyroid epithelial cells were capable of presenting a non-thyroidal antigen to antigen specific T-cell hybridomas, but were unable to present antigen to primed LNC. These results taken together suggest that thyroid epithelial cells, although they possess class II which is essential for presentation they lack further costimulatory molecules to present to a cell requiring more stringent activation requirements.

We know that thyroid epithelial cells have been shown to express adhesion molecules such as ICAM-1 (Weetman *et al.*, 1990a) and that they also produce IL-1 (Ebner *et al.*, 1987) which is also important in costimulation of T-cells. Recent discoveries regarding the CD80 family of molecules suggest the CD80 (B7.1) is a vital second signal that T-cells require to be able to fully respond to antigen. This is expressed by professional APC's following activation, there is no evidence to date that B7 is expressed by TEC's. Perhaps more importantly, B7.2 has been shown to be constitutively expressed on monocytes prior to activation and seems to be a critical molecule in the initiation of an immune response within the first 24h; B7.1 seems to be important in the maintenance of that immune response from 24h onwards. As TEC's have been shown to express and produce a number of molecules important for the costimulation of T-cells, it is possible that they could express B7.1; however to initiate an immune response they would have to express B7.2 constitutively. The weight of evidence suggests that thyroid epithelial cells are not initiators of anti-thyroidal T-cell responses. Thyroid infiltrating lymphocytes are often surrounded by areas

of class II expression suggesting that the class II expression is as a result in the local production of T-cell cytokines such as IFN-γ and TNF-α (Hamilton *et al.*,1991) and was not present previously. This is confirmed by the lack of class II expression in the OS chicken thyroid prior to development of thyroiditis (Wick *et al.*, 1984).

It is clear from the experiments shown in this thesis that TEC's are able to present thyroidal peptide antigens to T-cell hybridomas, however we have been unable to demonstrate that ability for activated T-cells *in vitro*. The *in vitro* situation is very artificial and the *in vivo* conditions of proximity of cells to each other and the local cytokine environment cannot easily be reproduced. Unfortunately due to time constraints we were unable to look at the effect of the addition of exogenous costimulatory cytokines or the expression of CD80 molecules on the thyroid cell surface; those experiments would be important to complete to enable us to fully assess the antigen presentation capacity of thyroid cells whilst imitating local conditions. If TEC's do not express CD80 molecules it would be interesting to do transient transfections to see if expression up-regulates presentation capacity, or whether B7 costimulation provided on third party cells would assist in antigen presentation to primed LNC.

The presentation of peptides to hybridomas suggests that in ongoing pathogenesis where T-cells have become activated against thyroidal antigens for one reason or another; the immune response has the potential to be enhanced by the TEC's due to local production of cytokines and subsequent class II expression. The natural breakdown of thyroglobulin in the vicinity of the thyroid may liberate peptides, some of which may contain T4 or T3 prior to the final liberation of the hormones. Also the liberation of Tg as a result damage to the thyroid structure by infiltrating cells may allow the local generation of peptide. These peptides then have the potential to compete with other non-immunogenic peptides for class II molecules and so prolong T-cell activation and ultimately thyroidal damage. Peptides may also be presented in the peripheral nodes as a result of thyroglobulin breakdown and allow T-cell activation. It is not known what the external stimulus is that is able to break the natural peripheral self tolerance of T-cells to thyroglobulin epitopes.

Clearly iodine plays a role but there seem to be many other factors which are contributory. Within the general population there is a sub-population of individuals that are predisposed to the development of autoimmunity by virtue of their HLA type and therefore their capacity to present pathogenic epitopes. Those pathogenic epitopes probably do not have to be iodinated given the evidence that some susceptible strains of H-2^k mice are able to develop EAT; but are unable to recognise thyroxine containing peptides. There is evidence that iodine may play a role in the development of disease, possibly be allowing the increased iodination of Tg and therefore increasing the generation of fully iodinated epitopes, however it has already been mentioned that these epitopes are probably not the only pathogenic epitopes.

As yet the trigger for the development of autoimmune thyroid disease in the human is unknown; in EAT the trigger is Tg. Immunisation has the effect of breaking self tolerance; in spontaneous models of the disease it appears that this self tolerance is broken possibly by a lack of regulatory cell function; this leads us to suppose that in the human condition an effective "immunisation" with Tg or TPO would also lead to the breaking of self tolerance in those already susceptible individuals. This still does not solve the puzzle as to what immunises the system and leads to a breakdown in tolerance. Molecular mimicry has often been postulated as mechanism by which T-cells involved in the development of many autoimmune diseases can become activated. However, given the uniqueness of the Tg molecule and its iodination state, it is difficult to envisage any environmental microorganisms that would on breakdown into peptide components, be able to stimulate thyroxine responsive T-cells.

The development of autoimmune thyroiditis in man requires the activation of multiple cell types such as Tc cells and Th cells and one of the activating determinants namely thyroxine containing peptide is present in the periphery as a result of increased iodine in the diet. What happens if the individual is of a MHC type capable of presenting that peptide and Tc cells become activated by the presence of non-iodinated peptide determinants which may be part of a microorganism? It has been shown by Gautam *et al.*, (1994) that a 11 mer peptide capable of causing EAE can be extensively substituted with alanine residue leaving only six of the original residues and still cause EAE. Only four native residues are required to stimulate EAE T-cells. The MHC and TCR residues alone, as long as they are in the correct configuration, can be recognised when presented by MHC to T-cells. The authors show that this configuration of amino acids is found in the published sequences of proteins from nineteen microorganisms, including measles virus, *E. Coli,* influenza, EBV, Coxsackie virus and Candida *sp* among others; all of these are common environmental microorganisms. If this can occur for peptides involved in the induction of EAE is it not entirely possible that other common environmental organisms contain thyroiditogenic sequences, that on clinical or sub-clinical infection are able to cross react with our own thyroid antigen reactive T-cells? It is highly unlikely that microorganisms would produce peptides that contain iodinated residues; but they may be responsible for initiating an immune response to particular peptide sequences. If those sequences correspond to peptides produced in the human which also happen to contain T4 residues; it is possible that as a result of epitope spread, this may subsequently generate an immune response to a thyroxine containing peptide.

In summary, we have been able to identify one T-cell peptide in the mouse that may go some way to explaining the involvement of dietary iodine in the pathogenesis of autoimmune thyroid disease in the human; from other mouse data it is clear the other pathogenic epitopes may also be involved in disease development. It is clear that thyroid

epithelial cells are able to present peptide antigens when expressing class II molecules; but the role that this plays in the development or perpetuation of the disease is open to debate. Although Tg has been shown to be an important epitope in the induction of EAT, TPO has been shown to be involved in the development of EAT and may be important in the development of thyroiditis in the human. There are obviously a number of factors such as genetic susceptibility, dietary iodine and possibly environmental organisms which have a role to play in the development of the autoimmune condition. The work contained within this thesis has only been able to provide a small amount of information as to the potential cause of autoimmune thyroid disease; but as they say: "Rome wasn't built in a day!"

7.0 References.

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Appendix 1.(to accompany Figure:-5.1A)

The effect of culture period.

Table 1.(Figure 5.1A)

IFN-γ	Antigen µg/ml	CPM x10^{-3}	SEM
Day 5			
+	MTg 50	3.85	0.079
+	T4^{+}(2553) 5	175.5***	4.8
+	NaI 1 µM	7.76***	0.498
+	Med	4.13	0.121
-	MTg 50	3.95	0.158
-	T4^{+}(2553) 5	16.9	2.54
-	NaI 1 µM	5.39	0.32
-	Med	3.84	0.157
Day 12			
+	MTg 50	0.281	0.052
+	T4^{+}(2553) 5	4.78	0.288
+	NaI 1 µM	0.479	0.188
+	Med	0.359	0.149
-	MTg 50	0.388	0.015
-	T4^{+}(2553) 5	5.23	0.386
-	NaI 1 µM	0.439	0.02
-	Med	0.479	0.035

Table 2.

IFN-γ	Antigen µg/ml	CPM x10^{-3}	SEM
Day 5			
+	MTg 50	2.89	0.065
+	T4^{+}(2553) 5	15.23***	1.45
+	NaI 1 µM	2.97	0.089
+	Med	2.66	0.043
-	MTg 50	3.10	0.06
-	T4^{+}(2553) 5	6.87	0.096
-	NaI 1 µM	3.62	0.084
-	Med	3.01	0.035
Day 12			
+	MTg 50	0.56	0.026
+	T4^{+}(2553) 5	3.42	0.12
+	NaI 1 µM	0.49	0.066
+	Med	0.38	0.02
-	MTg 50	0.75	0.011
-	T4^{+}(2553) 5	3.18	0.24
-	NaI 1 µM	0.66	0.034
-	Med	0.14	0.028

Table 3.

	Antigen µg/ml	CPM x10^{-3}	SEM
Day 5			
+	MTg 50	0.141	0.024
+	T4^{+}(2553) 5	3.062**	0.102
+	Med	0.264	0.095
-	MTg 50	0.137	0.096
-	T4^{+}(2553) 5	0.936	0.364
-	Med	0.122	0.021
Day 12			
+	MTg 50	0.227	0.064
+	T4^{+}(2553) 5	1.789	0.156
+	Med	0.231	0.065
-	MTg 50	0.123	0.046
-	T4^{+}(2553) 5	1.960	0.167
-	Med	0.140	0.028

Appendix 2.(to accompany Figure:-5.1B).

The effect of culture medium.

Table 1. (Figure 5.1B).

IFN-γ	Antigen µg/ml	CPM x10^{-3}	SEM	CPM x10^{-3}	SEM
		TEC 5%		TEC 5% + suppl	
+	T4+(2553) 5	18.27	0.090	19.77	2.3
-	T4+(2553) 5	8.47	1.275	6.71	0.61
+	Med	0.36	0.042	0.86	0.043
-	Med	0.31	0.054	1.04	0.042

Table 2.

IFN-γ	Antigen µg/ml	CPM x10^{-3}	SEM	CPM x10^{-3}	SEM
		TEC 5%		TEC 5% + suppl	
+	T4+(2553) 5	10.15	1.02	9.22	0.86
-	T4+(2553) 5	4.79	0.78	5.15	1.23
+	Med	1.23	0.064	1.56	0.035
-	Med	1.88	0.085	1.63	0.047

Appendix 3 (to accompany Figure 5.2)

The presence of ATA does not consistently improve thyroid antigen recognition.

Table 1.(Figure 5.2a)

IFN-γ	ATA 1%	Antigen µg/ml	CPM x10^{-3}	SEM
+	-	MTg 50	0.588	0.071
+	+		0.631	0.057
+	-	T4^{+}(2553) 5	1.615	0.411
+	+		3.891***	0.367
+	-	NaI 1µM	0.648	0.182
+	+		0.768	0.059
+	-	Med	0.572	0.142
+	+		0.611	0.044
-	-	MTg 50	0.687	0.094
-	+		0.562	0.041
-	-	T4^{+}(2553) 5	0.802	0.269
-	+		1.089	0.103
-	-	NaI 1µM	0.640	0.120
-	+		0.930*	0.092
-	-	Med	0.462	0.048
-	+		0.572	0.044

Table 2.(Figure 5.2b)

IFN-γ	ATA 1%	Antigen µg/ml	CPM x10^{-3}	SEM
+	-	MTg 50	10.96	0.37
+	+		10.33	0.209
+	-	T4^{+}(2553) 5	45.77	3.5
+	+		44.42	2.66
+	-	NaI 1µM	17.09	0.45
+	+		15.91	0.52
+	-	Med	13.96	0.25
+	+		14.33	0.508
-	-	MTg 50	14.98	0.487
-	+		15.13	0.555
-	-	T4^{+}(2553) 5	18.72	1.62
-	+		22.56	1.88
-	-	NaI 1µM	13.37	0.32
-	+		14.55	0.34
-	-	Med	11.8	0.27
-	+		12.61	1.25

Table 3.

IFN-γ	ATA 1%	Antigen µg/ml	CPM x10^{-3}	SEM
+	-	MTg 50	2.48	0.074
+	+		2.282	0.16
+	-	T4^{+}(2553) 5	9.236	0.152
+	+		8.645	0.16
+	-	NaI 1µM	2.947	0.079
+	+		3.386	0.187
+	-	Med	2.09	0.026
+	+		3.639	0.349
-	-	MTg 50	3.124	0.202
-	+		3.890	0.326
-	-	T4^{+}(2553) 5	2.88	0.14
-	+		3.144	0.311
-	-	NaI 1µM	2.45	0.062
-	+		3.002	0.3
-	-	Med	2.884	0.085
-	+		2.984	0.008

Table 4.

IFN-γ	ATA 1%	Antigen µg/ml	CPM x10^{-3}	SEM
+	-	MTg 50	2.004	0.126
+	+		2.117	0.074
+	-	T4^{+}(2553) 5	11.45	0.310
+	+		9.728	0.496
+	-	NaI 1µM	2.555	0.042
+	+		2.904	0.128
+	-	Med	2.987	0.366
+	+		3.035	0.246
-	-	MTg 50	3.056	0.58
-	+		3.003	0.229
-	-	T4^{+}(2553) 5	3.01	0.142
-	+		2.755	0.114
-	-	NaI 1µM	2.459	0.16
-	+		2.519	0.194
-	-	Med	2.048	0.051
-	+		2.643	0.156

Appendix 4 (to accompany Figure 5.3)

The presence of TSH does not improve thyroid antigen recognition.

Table 1.(Figure 5.3)

IFN-γ	TSH	Antigen µg/ml	CPM x10^{-3}	SEM
+	-	MTg 50	0.38	0.001
+	+		0.537	0.041
+	-	MTg 500	0.625	0.024
+	+		0.4	0.037
+	-	T4$^+$(2553) 5	16.23	0.124
+	+		16.42	0.078
+	-	NaI 1µM	0.721	0.027
+	+		1.926	0.227
+	-	Med	0.639	0.027
+	+		0.503	0.015
-	-	MTg 50	0.348	0.002
-	+		0.35	0.024
-	-	MTg 500	0.35	0.036
-	+		0.421	0.029
-	-	T4$^+$(2553) 5	7.23	0.399
-	+		5.023	0.696
-	-	NaI 1µM	0.589	0.038
-	+		1.074*	0.066
-	-	Med	0.329	0.017
-	+		0.473	0.011

Table 2.

IFN-γ	TSH	Antigen µg/ml	CPM x10^{-3}	SEM
+	-	MTg 50	0.852	0.125
+	+		0.721	0.089
+	-	MTg 500	0.83	0.054
+	+		0.802	0.103
+	-	T4$^+$(2553) 5	21.25	2.054
+	+		20.19	3.21
+	-	NaI 1µM	0.63	0.052
+	+		0.79	0.15
+	-	Med	0.723	0.057
+	+		0.835	0.1
-	-	MTg 50	0.899	0.165
-	+		0.832	0.122
-	-	MTg 500	0.916	0.201
-	+		0.957	0.155
-	-	T4$^+$(2553) 5	1.232	0.222
-	+		1.347	0.033
-	-	NaI 1µM	0.814	0.058
-	+		0.788	0.104
-	-	Med	0.694	0.089
-	+		0.718	0.119

Appendix 5 (to accompany Figure 5.4)

Thyroid epithelial cells cannot present antigen to primed lymph node cells.

Table 1a. (Figure 5.4a)

In vitro antigen µg/ml	IFN-γ	CPM x10^{-3}	SEM
MTg 50	+	1.13	0.37
	-	1.14	0.17
T4$^+$(2553) 5	+	1.21	0.33
	-	1.21	0.28
NaI 1 µM	+	1.25	0.25
	-	1.19	0.18
Med	+	1.22	0.2
	-	1.18	0.16

Table 1b. (Figure 5.4b)

In vitro antigen µg/ml	IFN-γ	CPM x10^{-3}	SEM
MTg 50	-	0.85	0.17
	+	0.85	0.15
T4$^+$(2553) 5	-	1.19	0.22
	+	0.82	0.09
NaI 1 µM	-	1.33	0.16
	+	0.92	0.18
Med	-	1.04	0.18
		0.96	0.11

Table 1c. (Figure 5.1c).

In vitro antigen µg/ml	Immun	CPM x10^{-3}	SEM
MTg 50	MTg	4.96***	0.86
	T4$^+$(2553)	1.63	0.22
T4$^+$(2553) 5	MTg	9.33***	0.55
	T4$^+$(2553)	7.62***	0.70
NaI 1 µM	MTg	1.06	0.21
	T4$^+$(2553)	1.00	0.19
Med	MTg	0.98	0.15
	T4$^+$(2553)	0.93	0.12

Table 2a. (TEC presentation to MTg immunised LNC)

In vitro antigen μg/ml	IFN-γ	CPM x10^{-3}	SEM
MTg 50	+	3.26	0.143
	-	3.58	0.120
T4$^+$(2553) 5	+	4.05	0.204
	-	3.98	0.183
NaI 1 μM	+	3.02	0.098
	-	2.84	0.063
Med	+	2.99	0.015
	-	3.16	0.106

Table 2b. (spleen cell controls).

In vitro antigen μg/ml	Immun	CPM x10^{-3}	SEM
MTg 50	MTg	17.91***	2.02
	T4$^+$(2553)	6.55	1.06
T4$^+$(2553) 5	MTg	22.39***	1.97
	T4$^+$(2553)	26.4***	2.69
NaI 1 μM	MTg	4.24	0.99
	T4$^+$(2553)	4.93	1.25
Med	MTg	4.86	0.56
	T4$^+$(2553)	5.07	1.23

9.0 Publications.

Identification of a Thyroxine-Containing Self-Epitope of Thyroglobulin Which Triggers Thyroid Autoreactive T Cells

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Summary

Although thyroglobulin (Tg), the thyroid prohormone, is well known as a T cell dependent autoantigen in human and experimental autoimmune thyroid disease, very little is known about the molecular basis of Tg recognition by T cells. In this paper, we have characterized the epitopes recognized by two clonotypically distinct, murine Tg autoreactive T cell hybridomas, CH9 and ADA2. In vitro iodination of a Tg preparation which was deficient in in vivo organified iodine was first used to confirm our previous observation that these T cells recognize iodination-related epitopes in the Tg molecule. Affinity chromatography of tryptic peptides derived from normally iodinated human Tg revealed that these epitopes were exclusively located in thyroxine (T4) containing peptides. Through the use of synthetic T4-containing peptides, representing the four major hormonogenic sites in Tg, we demonstrated that both CH9 and ADA2 recognize an epitope containing the T4 at position 2553 in human Tg. Sets of overlapping 5mer to 12mer peptides around this T4 showed that the most potent peptide was a 9mer beginning at Asp 2551. The T4 was shown to be a critical residue, since its replacement with any of the 20 naturally occurring amino acids produced only nonstimulatory peptides. Since the T cell hybridomas could also be stimulated by major histocompatibility complex class II positive (interferon-γ-treated) thyroid epithelial cells in vitro, and their parent T cell lines can induce thyroiditis on adoptive transfer, the T4-containing Tg sequence described here is implicated as a pathogenic epitope in murine thyroid autoimmunity.

The production of the thyroid hormones thyroxine (T4),[1] tri-iodothyronine (T3) and reverse T3 (rT3) is dependent on the organification of iodine into thyroglobulin (Tg), the major protein product of the thyroid (1, 2). This involves thyroid peroxidase catalyzed iodination of tyrosine residues in Tg to form mono- and di-iodotyrosines and their subsequent crosslinking to form the iodothyronines T3 and T4. These mature Tg molecules are stored in a colloidal form in the lumen of thyroid follicles. Secretion of T4 and T3 involves the endocytosis and subsequent proteolysis of colloidal Tg, which releases the hormone residues for diffusion into the circulation. The recent cloning of the genes coding for Tg from several species has allowed the precise localization of the four main hormonogenic sites within the molecule (3–5). In human Tg, tyrosines at positions 5, 2553, 2567 and 2746 of the 2748 amino acid monomer may be modified to form T4 or T3 residues. Under conditions of limited iodine availability, hormonogenesis occurs preferentially at position 5 near the N-terminus (6, 7). Factors other than iodine levels which may regulate the degree of hormonogenesis at the other sites are not clear. However, some evidence suggests that position 2746 may be preferentially converted to T3 rather than T4 (8).

Tg has been known for over three decades as an autoantigen in human and experimental autoimmune thyroid disease (1, 9–11). Epidemiologic and experimental evidence has indicated that the autoantigenicity of Tg may be influenced by its iodine content. For example, raised dietary iodine levels in humans (12–14) and experimental animals (15–19) has been correlated with an increase in the incidence and severity of autoimmune thyroid lesions. In chickens, highly iodinated

[1] *Abbreviations used in this paper:* HTg, human thyroglobulin; MTg, mouse thyroglobulin; TEC, thyroid epithelial cells; Tg, thyroglobulin; T3, tri-iodothyronine; T4, thyroxine; rT3, reverse tri-iodothyronine; TSH, thyroid stimulating hormone.

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Tg has been shown to induce stronger autoantibody responses than poorly iodinated Tg (20), while in mice, inhibition of the peroxidase reaction with aminotriazole produces very poorly iodinated Tg which fails to elicit autoimmune thyroiditis in high responder CBA/J mice (21). To date, it is not clear which of the many iodination sites in Tg are responsible for these effects on Tg antigenicity or how they are mediated.

However, our recent observation that two clonotypically distinct autoreactive murine T cell hybridomas recognized iodination-dependent epitopes in Tg (21) provided a clue to the molecular basis of Tg autoantigenicity. We now report that both of these T cells recognize a T4-containing peptide centered on the hormonogenic site at residue 2553 in human Tg, and that this epitope is expressed on the surface of mouse thyroid epithelial cells (TEC) cultured with IFN-γ.

Materials and Methods

Thyroglobulin Preparations. Mouse Tg (MTg) was prepared from pooled thyroids and human Tg (HTg) from individual post-mortem thyroids as previously described (22). The levels of organified iodine and thyroid hormone residues in Tg preparations were determined as described elsewhere (23).

Thyroglobulin Iodination. HTg of low iodine content (TgMC, 0.08 T4 residues/mole) was iodinated with NaI by the Iodogen method (24), with reaction times of 10–80 min (transferring the mixture to fresh iodogen-coated tubes every 10 min) to produce preparations (TgMC1-6) differing in their levels of organified iodine and T4 residues.

Preparation of Tg Peptides. HTg was reduced and alkylated with iodoacetamide in 8 M urea (25), dialyzed to remove the urea, digested with TPCK-Trypsin (Sigma Chemical Co., Poole, UK) at an enzyme:substrate ratio of 1:50 in 0.1 M ammonium bicarbonate for 4 h. The digest was lyophilized and redissolved in 20 mM sodium phosphate, pH 7.4 to 1 mg/ml and passed through an affinity matrix consisting of rabbit anti-T4 anti-bodies (Miles, Stoke Poges, UK) coupled to tosyl activated Sepharose 4B (26). Unadsorbed peptides ($T4^-$) were collected. Adsorbed peptides ($T4^+$) were recovered by elution with 0.1 M ammonia in 10% (v/v) ethanol, lyophilized and reconstituted to the original column loading volume in 20 mM sodium phosphate, pH 7.4. The concentrations quoted in Results for the $T4^+$ peptides represent those of equivalent dilutions of unseparated tryptic peptides.

Synthetic peptides varying in length from 5 to 12 amino acids were produced on derivatized polyethylene pins as described previously (27).

T Cells. The generation, characteristics and maintenance of two autoreactive murine Tg-specific T cell hybridomas, CH9 and ADA2, have been described elsewhere (21, 28, 29). Briefly, both were obtained by fusion of BW5147 cells with mouse Tg-specific, I-A^k restricted T cell lines; CH9 was generated from the noncloned line MTg12B, whereas ADA2 was derived from the T cell clone MTg9B3. The T cell receptors expressed by CH9 and ADA2/MTg9B3 have been shown to be distinct by both T cell receptor β chain restriction fragment polymorphism (29) and their differential reactivity with Tgs prepared from thyroids of different species (28). Both hybridomas produce IL-2 and not IL-4 on activation with specific antigen (Champion, B.R., unpublished observations). CH9 and ADA2 were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, nonessential amino acids (1x; Flow Laboratories, Irvine, Scotland), 50 U/ml benzylpenicillin and 50 μg/ml streptomycin (RPMI/10% FCS). MTg12B and MTg9B3 were maintained by regular cycles of activation with mouse Tg and antigen presenting cells (APCs: 3000R irradiated syngeneic spleen cells), followed by expansion in IL-2 containing medium (DMEM supplemented as for RPMI/10% FCS) as described elsewhere (30). The IL-2–dependent cell line CTLL (31) was maintained in RPMI/10% FCS supplemented with 1% phorbol myristate acetate-stimulated EL-4 supernatant as a source of IL-2.

IL-2 Release Assay. Specific activation of CH9 and ADA2 was measured by their ability to release IL-2 in response to different stimuli in the presence of APCs as described elsewhere (29). Briefly, $1–2 \times 10^5$ hybridoma cells were cultured with irradiated (3000 R) splenic APCs (5×10^5 unless otherwise stated) in the presence of test antigens in 200 μl RPMI/10% FCS. After 24-h, 100 μl aliquots of supernatant were tested for their IL-2 content by their ability to support the proliferation of CTLL cells (10^4/well). Proliferative responses were assessed by the incorporation of either ^{3}H-thymidine or ^{125}I-deoxyuridine as indicated in the Results. We have previously shown that both CH9 and ADA2 produce relatively low levels of IL-2 which are always nonsaturating for the CTLL cells when compared with standard IL-2 preparations (29). Thus, results are simply expressed as incorporation of radiolabel by the CTLL cells (arithmetic mean cpm ± SD).

Proliferative Responses of T Cell Lines. Specific activation of MTg9B3 cells by antigen was directly assessed by the incorporation of radiolabel during the last 18 h of a 3-d culture as previously described (30). Results are expressed as mean cpm ± SD.

Thyroid Epithelial Cell (TEC) Cultures. Modification of the methods of Creemers et al. (32) and Chiovata et al. (33) were used to establish cultures of mouse TEC. Pooled thyroids from CBA/Ca ($H\text{-}2^k$) mice were minced with fine scissors in 5 ml Hank's balanced salt solution (HBSS) containing 1.5 mg/ml collagenase (Sigma type IV; Sigma Chemical Co.) and incubated for 1–2 h at 37°C. At regular intervals, the mixture was vigorously pipetted to aid disruption of the follicles. The suspension was then diluted to 15 ml in HBSS containing 20% FCS. Following centrifugation ($200g_{av}$, 10 min), the cell pellet was resuspended and washed two times in HBSS/10% FCS. The cells were then suspended in culture medium (RPMI/10% FCSL) containing sodium iodide (1 μM), hydrocortisone (10^{-8} M), bovine insulin (100 IU/ml), bovine transferrin (5 μg/ml) and HEPES (25 mM) as additional supplements. Because of clumping it proved difficult to accurately count the TECs before culture. Therefore, cells prepared from 10–12 thyroids were used to seed one complete 96-well flat-bottomed microtiter plate (200 μl/well). After 1 d of culture, nonadherent cells were removed by extensive washing and the adherent cells recultured in complete medium containing 1% FCS. After 2 d culture, some wells received rat IFN-γ (100 U/ml). At day 5, cells were washed three times with T cell hybridoma culture medium and CH9 cells (10^5/well) added for 24 h. Cell-free supernatants were then tested for IL-2 as described above. These TEC culture conditions were found to be optimal for the induction of I-A^kMHC class II molecules on the TEC surface by IFN-γ, as assessed by standard immunohistochemical techniques with specific antibodies (not shown). Microscopic examination of the TEC monolayers before addition of hybridoma cells showed them to be free of mononuclear cells.

Results

Autoreactive Tg-specific Cloned T Cells Recognize Iodination-Dependent Epitopes of Tg. We have previously reported that

two clonally distinct Tg-specific, I-A^k-restricted autoreactive T cell populations, represented by the hybridomas CH9 and ADA2 (and the parent clone of ADA2, called MTg9B3), only recognize Tg which is sufficiently iodinated (21). These clones have also been shown to have distinguishable epitope specificities by their differential responsiveness to a panel of Tgs prepared from different species (28). Both could be cross-stimulated by human Tg, but recognized bovine Tg weakly or not at all (28).

To further evaluate the critical role of iodination in recognition of Tg by these T cells, the Iodogen method was used to generate iodotyrosine and iodothyronine residues in a poorly iodinated human Tg preparation (TgMC). Different incubation conditions were used to prepare samples (TgMC1-6) which differed in their total iodine content and their T4 levels. The response of MTg9B3 to these Tg preparations is illustrated in Fig. 1. In contrast to the lack of response to the poorly iodinated TgMC, MTg9B3 cells responded well to in vitro iodinated preparations TgMC1-5. The TgMC6 preparation failed to stimulate MTg9B3 cells. Similar results were obtained with the hybridomas ADA2 and CH9 (not shown). The lack of response to the most heavily iodinated preparation (TgMC6), found with all the cells, suggests that excessive iodination in some way blocks processing and presentation of the critical epitope.

CH9 and ADA2/MTg9B3 Recognize Thyroxine-Containing Tg Tryptic Peptides. To test the importance of T4 residues to the antigenic site(s) recognized by CH9 and ADA2/MTg9B3, human Tg was reduced and alkylated followed by trypsin digestion and affinity chromatography of the tryptic peptides on an anti-T4 column to produce T4-depleted ($T4^-$) and T4-enriched ($T4^+$) peptide preparations. As previously reported (1), CH9 responded as well to tryptic peptides as to native Tg or reduced/alkylated Tg, whereas ADA2 (and MTg9B3, not shown) responded less well to the trypsin digest (Fig. 2). Affinity chromatography effected a complete compartmentalization of the stimulatory peptides for both T cell populations into the $T4^+$ peptide pool. Although peptides lacking T4 may have non-specifically adsorbed to the column to become included in the $T4^+$ peptide pool, this result indicated that the epitope(s) is associated with a hormonogenic site (as opposed to a sequence containing iodotyrosines, or dehydroalanine produced incidentally during peroxidase-catalyzed Tg modification [1, 34]).

CH9 and ADA2/MTg9B33 Recognize Closely Related Epitopes of Human Tg Containing T4 at Residue 2553. The results portrayed in Fig. 2 indicated that the epitopes recognized by CH9 and ADA2 involved one or more of the four hormonogenic sites in Tg (1, 2), or were located close to one of these sites. The amino acid sequences of these 4 sites in human Tg are shown in Fig. 3. To determine which, if any, of these T4 residues was involved in forming the epitopes for CH9 and ADA2/MTg9B3, overlapping sets of 12-amino acid poly-

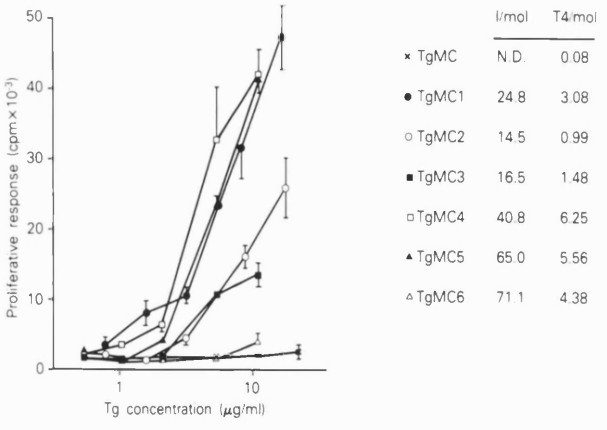


Figure 1. In vitro iodination of human Tg of low intrinsic iodine content generates the epitope recognized by the autoreactive, Tg-specific mouse T cell clone, MTg9B3. Aliquots of a preparation of human Tg with low T4 content (TgMC) were iodinated in vitro to produce the preparations TgMC1-6, whose iodine and T4 contents are shown. Proliferative responses (mean ± SD) of triplicate cultures of MTg9B3 cells to those different Tg preparations are shown as the incorporation of ^{125}I-deoxyuridine (cpm × 10^{-3}). The same response pattern was observed for the T cell hybridomas CH9 and ADA2 (not shown).

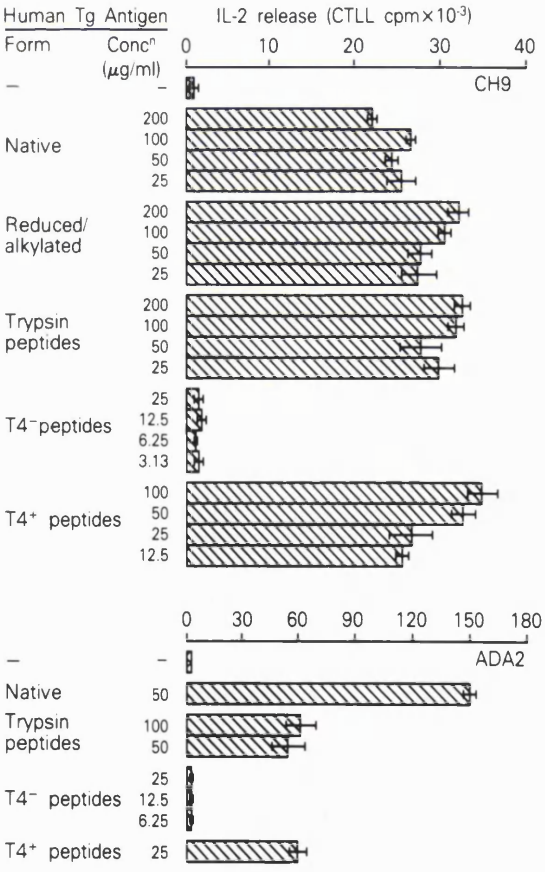


Figure 2. Tg autoreactive T cell hybridomas recognize T4-bearing human Tg peptides. The responses (IL-2 release) of autoreactive T cell hybridomas CH9 and ADA2 to different human Tg antigen preparations are shown. Separation of trypsin-cleaved Tg peptides according to their T4 content by affinity chromatography clearly showed that both hybridomas recognize epitopes within the T4-containing ($T4^+$) but not the depleted ($T4^-$) fraction. IL-2 activities released were assessed in a CTLL proliferation assay as described in Materials and Methods. Results (mean ± SD) are expressed as ^{125}I-deoxyuridine incorporation (cpm × 10^{-3}) of triplicate CTLL cultures.

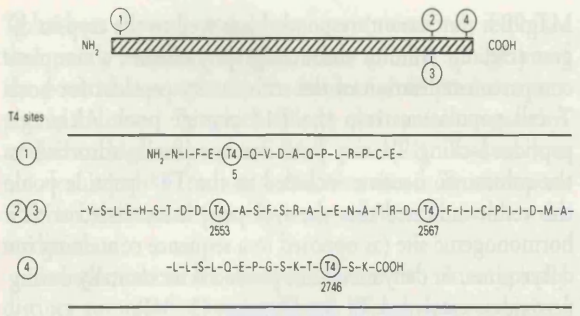


Figure 3. The positions of the four hormonogenic sites and the surrounding amino acids in the primary sequence of human Tg[3]. Amino acids are represented in single letter code, and the numbers show the position of the hormonogenic sites within the Tg primary sequence.

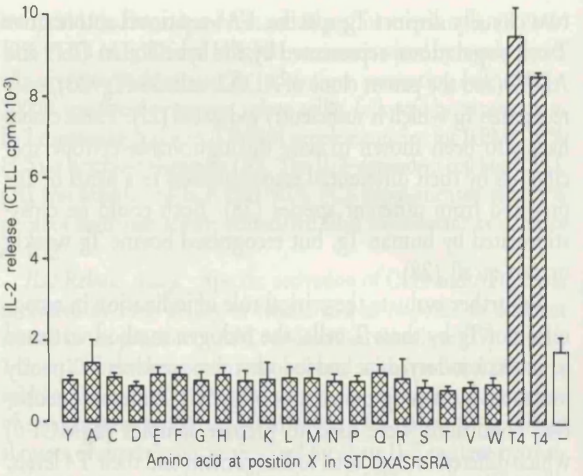


Figure 5. T4 is essential for peptide recognition by CH9 cells. Responses of CH9 to 11mer peptides (2.5 μg/ml) of sequence STDDXASFSRA, where X is the amino acid or T4 indicated, are shown. The response of CH9 with no added peptide is also shown (–). Similar results were obtained with the 9mer and 10mer sequences TDDXASFSRA and DDXASFSRA (not shown). Responses were assessed as described in Fig. 2.

peptides centered on each of the four hormonogenic sites in the primary sequence of human Tg were synthesized. These peptides were then tested for their ability to stimulate CH9 and ADA2 cells. The results (illustrated in Fig. 4) clearly demonstrated that both CH9 and ADA2 recognize an epitope involving the T4 residue at position 2553. The sequence surrounding this residue corresponds to a potential T cell epitope based on two predictive algorhythms (35, 36). Replacement of T4 in the sequence with any of the 20 naturally occurring amino acids (including tyrosine) abrogated the capacity to stimulate both CH9 (Fig. 5) and ADA2 (not shown), indicating that this T4 is a critical residue for both T cell hybridomas.

To delineate the boundaries of this epitope, we challenged the T cell hybridomas with a series of 5 to 12 mer peptides centered on the T4 at position 2553. T4-containing peptides

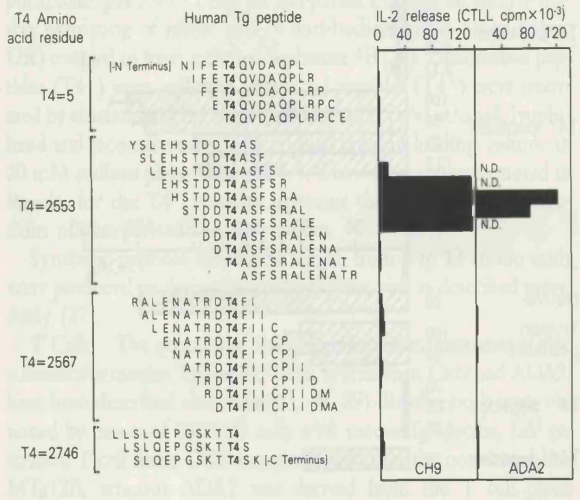
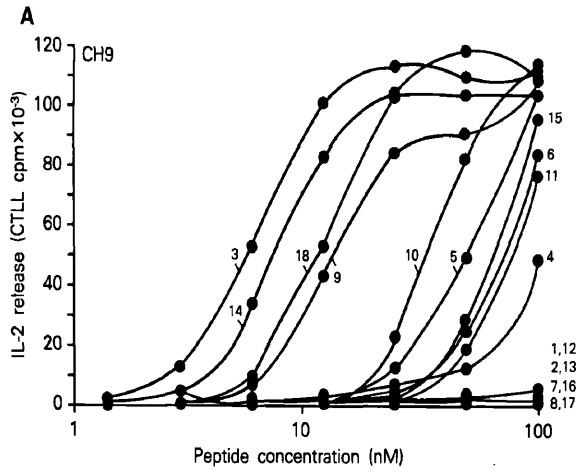


Figure 4. CH9 and ADA2 both recognize an epitope centered on the T4 residue at position 2553 in human Tg. Sets of overlapping T4-containing 12mer peptides, representing the four hormonogenic sites illustrated in Fig. 3, were synthesized on derivatized polyethylene pins as previously described (27). The responses of CH9 and ADA2 to these peptides (at 2.5 μg/ml) are shown. The amino acids in each peptide are presented in one-letter code. Responses were assessed as described in Fig. 2, except that the incorporation of ^{3}H-thymidine was used to assess the proliferation of CTLL cells.

of 8 amino acids or less were all non-stimulatory for either T cell hybridoma at concentrations up to 1.25 μM (not shown), as were peptides beginning at Asp 2552 (see Fig. 4). This indicates that the smallest fully effective epitope is a T4-containing 9mer. At present we cannot rule out the possibility that a smaller peptide could stimulate at much higher concentrations. Fig. 6 shows example dose curves for both CH9 and ADA2 stimulated with different 9 to 12mer peptides and summarizes the relative stimulatory capacity of these peptides in terms of the concentration required to achieve half-maximal stimulation (ED_{50}). Deletion of Ala 2559 from the C-terminus of the peptide abrogated their ability to stimulate CH9, but only diminished their activity with ADA2 by 20–30-fold. Elongation of the peptides C-terminal to Ala 2559 consistently diminished the stimulatory capacity for both T cells, whereas addition of residues N-terminal to Asp 2551 had no appreciable effect on antigenic potency. These observations, coupled with previous data using a panel of Tgs from different species (28), indicate that CH9 and ADA2 recognize the same T4-containing epitope in Tg, but have subtly different structural requirements for optimal stimulation. Comparison of the published primary sequences of human (3), rat (4), and cow (5) Tg in this region revealed that the maximally stimulating 9mer (boxed in Fig. 7) is identical in all three species.

Mouse Thyroid Epithelial Cells Cultured with IFN-γ Express the Epitope Recognized by CH9. To determine whether the epitope described above can be expressed by thyroid cells, we cocultured CH9 with primary cultures of syngeneic TEC, without added Tg. TEC precultured with IFN-γ, using conditions which induce MHC class II expression (not shown) and increase Tg release (1), were able to stimulate IL-2 production by CH9 cells (Fig. 8). The level of response was generally low, but similar to that triggered by comparable numbers



C

Peptide	Sequence	ED_{50} (nM) CH9	ADA2
2	E H S T D D T4 A S F S R	>1250	30
7	E H S T D D T4 A S F S	>1250	>500
3	H S T D D T4 A S F S R A	6	2.5
8	H S T D D T4 A S F S R	>1250	48
12	H S T D D T4 A S F S	>1250	>500
4	S T D D T4 A S F S R A L	100	72
9	S T D D T4 A S F S R A	14	3.4
13	S T D D T4 A S F S R	>1250	85
16	S T D D T4 A S F S	>1250	>500
5	T D D T4 A S F S R A L E	52	23
10	T D D T4 A S F S R A L	34	14
14	T D D T4 A S F S R A	8	2.2
17	T D D T4 A S F S R	>1250	70
6	D D T4 A S F S R A L E N	70	32
11	D D T4 A S F S R A L E	75	32
15	D D T4 A S F S R A L	62	25
18	D D T4 A S F S R A	12	7.7
1	P L R Q G G G G G G G G	>1250	N.T.

Figure 6. Responses of CH9 and ADA2 to T4-containing peptides of different length representing the hormonogenic site at position 2553 of human Tg. (*A* and *B*) Representative dose-curves of CH9 and ADA2 responses to the peptides defined in C. (*C*) The responses of CH9 and ADA2 to the T4-containing peptides shown are represented as the concentration required to stimulate a half-maximal response (ED_{50}). Peptides of 5, 6, 7, or 8 amino acids containing the T4 residue were all nonstimulatory (not shown). Results represent mean ^{3}H-thymidine incorporation by triplicate cultures of CTLL cells. NT = not tested.

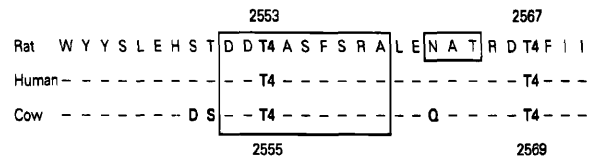


Figure 7. Comparison of the amino acid sequences of rat[4], human[3] and cow[5] Tg around the hormonogenic site at position 2553 (2555 in cow Tg). The minimal 9mer sequence recognized by CH9 and ADA2 is boxed, as is the N-glycosylation site at position 2562 in human and rat Tg which is missing in cow Tg.

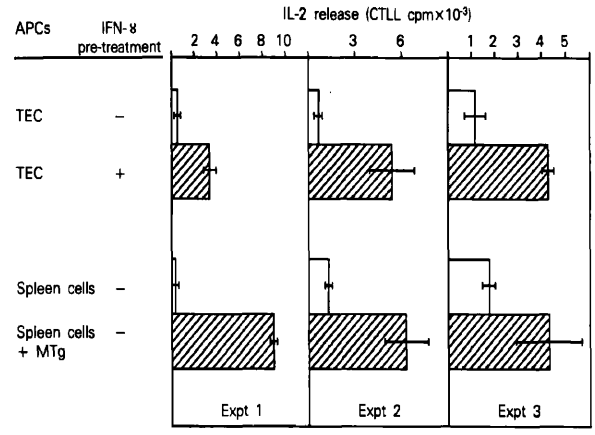


Figure 8. The epitope recognized by the T cell hybridoma CH9 can be expressed by IFN-γ-treated syngeneic thyroid epithelial cells (TEC). Three experiments demonstrating the activation of CH9 by TEC precultured with (or without) IFN-γ, to induce MHC class II expression, are shown. Responses to mouse Tg (MTg) presented by comparable numbers of splenic antigen presenting cells are shown for comparison. Responses were assessed as described in Fig. 2.

of irradiated spleen cells in the presence of MTg. This indicates that the T4-containing epitope can be generated by TEC and expressed in association with I-A^k on their cell surface.

Discussion

In this paper we have shown that two independently derived autoreactive mouse T cell hybridomas both recognize (albeit slightly differently) the same T4-containing epitope in Tg. Although we have shown that the T4 residue is critical for recognition by these hybridomas, it is not yet clear whether it directly interacts with the T cell receptor or the I-A^k molecule (or both). This is a particularly intriguing question when one considers the extremely large molecular size of T4 compared with the rest of the 9mer peptide (molecular weight of 763 out of a total 1627 for the entire peptide). Competition studies and molecular modeling will be required to address this point.

A comparison of the amino acid sequences around this T4, at position 2553 in human Tg, with those around the equivalent site in rat and bovine Tg (the mouse Tg sequence is not available) provide interesting information regarding the recognition of this autoantigenic site by CH9 and ADA2 (Fig. 7). As previously reported (29), both CH9 and ADA2 were

generated as autoreactive mouse Tg-specific T cells which cross-reacted well with human and rat Tg, but were only weakly responsive to bovine Tg (and other members of the Artiodactyla family). However, the sequence containing the minimal stimulatory epitope defined above (boxed in Fig. 7) is identical in all three species. This observation suggests that sequences outside of the minimal epitope also play a critical role in generating stimulatory I-A^k/peptide complexes. Since position 2555 in bovine Tg has been shown to be one of the major hormonogenic sites (5), it is unlikely that the lack of reactivity of our T cells to this species of Tg is due to the lack of a T4 residue at this position (the bovine Tg used had normal T4 levels). A more plausible hypothesis is that residues outside the epitope influence the processing of Tg by antigen presenting cells. An alternative, but not mutually exclusive, explanation would be that the naturally processed Tg fragment is larger than the minimal epitope and that residues included in this fragment influence either the formation or activity of I-A^k/peptide complexes. The information in Fig. 7 presents two testable explanations for the low potency of bovine Tg. The replacement of Ser 2549 and Thr 2550 in human Tg with Asp and Ser, respectively, in bovine Tg might prevent MHC binding of a peptide containing these residues or remove/create critical proteolytic cleavage sites. Similar considerations could be given to the Gln for Asn substitution at position 2562; this substitution also removes an N-glycosylation site (known to be glycosylated in human Tg [37]) which could influence intracellular processing events.

The ability of IFN-γ stimulated TECs to activate the hybridoma CH9 without the addition of Tg indicates that the epitope described here can be functionally expressed by thyroid cells, at least in vitro. This observation appears to conflict with other reports (38–40) that mouse TEC will only present antigen to T cell lines or hybridomas following the induction of a poorly defined costimulatory activity. This discrepancy may simply reflect differences in the costimulator requirements of the cells used; some T cell hybridomas appear to lack a requirement for costimulatory signals since they can be stimulated by isolated MHC/peptide complexes (41). Alternatively, TECs may be particularly efficient at presenting the epitope described here since the antigen (Tg) is produced in relatively large amounts by the cells themselves. Ebner et al. (39) have shown that TEC could stimulate a class I (K^b) alloreactive T cell hybridoma, but not class II alloreactive cells. Although this most likely reflects differences in signalling requirements between class I- and II-restricted T cells, the different processing routes usually used to generate the epitopes recognized by these cells (42) could also be important. Class II-restricted T cells which recognize epitopes generated within the cell from endogenous antigens (the processing route primarily used by class I-restricted epitopes) have been described (43). We are, therefore, in the process of determining the antigen processing route used by TEC to present the T4-containing epitope recognized by CH9 cells. Whether or not thyroid cells can express the relevant epitope in vivo is not clear at present. However, in preliminary experiments, we have shown that the T cell line MTg12B (parental line to CH9) and the clone MTg9B3 can elicit thyroid lesions in irradiated CBA/J recipients, when transferred 3 days following activation with antigen in vitro (not shown). These observations, coupled with the previously described inability of T4-deficient Tg to elicit thyroiditis in CBA mice (21), indicate that T cell recognition of the T4-containing epitope of Tg described in this paper can have pathological consequences.

A striking feature of the autoantigenic T cell epitope described in this paper is that it is generated from Tg in the target organ by physiologically important post-translational mechanisms involved in the hormone-forming function of the gland. The production of this epitope will depend on the relative availability of iodine in the gland: under conditions of iodine deficiency, very little will be formed due to hormonogenesis occurring primarily at the N-terminal site (6, 7). Since some evidence suggests that circulating Tg may be poorly iodinated, particularly in neonates (44–46), T cell tolerance to the T4-containing epitope may not be effectively established. This could account for the ease of induction of both experimental tolerance (47, 48) and autoimmune thyroiditis (49) in mice. It may also help explain the ability of thyroid stimulating hormone (TSH) to induce tolerance to Tg in mice (48), since TSH administration has been reported to stimulate increases in the iodine content of circulating Tg (50). Although the importance of T4-containing T cell epitopes in human thyroid autoimmunity is unknown, our findings could provide a molecular basis for the link between dietary iodine levels and spontaneous thyroid disease, both in man (12–14) and in animals (15–19).

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Brief Definitive Report

A Thyroxine-containing Peptide Can Induce Murine Experimental Autoimmune Thyroiditis

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Summary

A synthetic peptide based on a sequence containing thyroxine at position 2553 in thyroglobulin (Tg), and already shown to be recognized by two clonotypically distinct murine Tg autoreactive T cell hybridomas, can trigger primed lymph node cells to transfer thyroiditis to naive recipients. Donor lymph node cells could be prepared from mice immunized either with intact mouse Tg or with this peptide itself. After a second exposure to the priming antigen in vitro, both these populations induced 100% thyroiditis in recipient animals. The importance of the T4 residue in the development of disease was demonstrated by the failure of Tg tryptic peptides depleted of T4 to stimulate pathogenic effectors in vitro, even when the lymph node cells had been taken from mice primed with whole Tg. We conclude that this T4-containing 12mer sequence is a major thyroiditogenic epitope in CBA/J mice although we cannot exclude the possibility that there are other pathogenic epitopes present in the whole Tg molecule.

Experimental autoimmune thyroiditis (EAT) is characterized by an inflammatory infiltrate of the thyroid gland. It has been shown that the cells comprising this infiltrate include macrophages and T cells, and in animal models of induced thyroiditis, the disease has been shown to be T cell dependent (1).

Thyroglobulin (Tg) a 660-kD glycoprotein, is synthesized in the thyroid by follicular epithelial cells, and tyrosine residues within the molecule are iodinated by thyroid peroxidase to produce the thyroid hormones tri-iodothyronine (T3) and thyroxine (T4). In human Tg, T3 and T4 residues may be formed from tyrosines at positions 5, 2553, 2567, and 2746.

There is evidence from animal models that the iodine content of Tg may influence its autoantigenicity (2, 3), and EAT induced in mice is much less severe if poorly iodinated Tg is used for immunization (4). Similarly, human studies have shown that raised dietary iodine correlates with an increase in the incidence and severity of autoimmune thyroid lesions (5).

In previous studies (6) using synthetic T4-containing peptides, we showed that two clonotypically distinct murine Tg autoreactive T cell hybridomas recognized an epitope containing T4 at position 2553 in human Tg. Sets of overlapping 5mer to 12mer peptides around this T4 showed that the most potent peptide was a 9mer beginning at Asp 2551, and replacement of the T4 with any of the 20 naturally occurring amino acids led to loss of antigenicity. Since the parent T cell lines of the hybridomas have been shown to induce thyroiditis on adoptive transfer, the T4-containing sequence appeared to be implicated as a pathogenic epitope in murine EAT. To confirm the pathogenic potential of this sequence, we have used the T4-containing 12mer to induce thyroiditis in vivo, and the results of these studies are the subject of this communication.

Materials and Methods

Preparation of Antigens, Mice, and Immunization Protocol. Mouse thyroglobulin (MTg) was prepared by extraction from pooled homogenized thyroids (Parkes outbred strain reared in our own laboratory) as previously described (7). CBA/J mice, 6–8 wk old, of either sex (NIMR, London, UK), were immunized either in the base of the tail with 50 μg MTg emulsified in CFA (MTg/CFA; H37Ra; Difco Laboratories, Detroit, MI) or with 50 μg MTg in PBS intravenously followed 3 h later by an intravenous injection of 20 μg LPS (MTg/LPS: *Salmonella enteridites;* Sigma Chemical Co., St. Louis, MO). Mice were boosted 7 d later with 25 μg MTg/CFA in both hind footpads or 50 μg MTg/LPS as previously.

Preparation of Tg Peptides. Peptides lacking thyroxine ($T4^-$)

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were prepared as described previously (6, 8, 9), and the synthetic peptide Ac-STDD(T4)ASFSRAL-NH_2, hereafter referred to as the T4(2553) peptide, was produced on a synthesizer (9050; Milligen/Biosearch, Burlington, MA) also as described previously (10).

Transfer of Thyroiditis. The popliteal lymph nodes from MTg/CFA-immunized mice or spleens from MTg/LPS-immunized mice were taken on day 16 and cultured for 72 h in petri dishes or flasks at 5 × 10^6/ml in RPMI 1640 (Gibco, Paisley, Scotland) with 5% FCS and other supplements, as described previously (6), together with MTg (40 μg/ml) or peptide (2.5 or 5 μg/ml) to activate and expand effector T cells. After washing, 2 × 10^7 viable lymph node cells or 3–5 × 10^7 spleen cells were injected intravenously into normal syngeneic recipients and their thyroids examined 14 d later.

Evaluation of Thyroid Infiltration. Thyroids were fixed in 4% phosphate-buffered formalin and sections stained with H and E. For each mouse, there are 12 possible scores (two lobes at six levels). 0, no infiltration; 1, any definite infiltration up to 20%; 2, between 20 and 50% infiltration; 3, between 50 and 75% infiltration; 4, gland totally infiltrated but follicles still discernible. The score for each mouse is the total divided by the number of observations (as occasionally, one lobe may be missing on some sections). The average thyroiditis for each group is the mean ± SE of all of the mice in the group.

Proliferation Assay. Mice were immunized once in both hind footpads with 50 μg MTg/CFA. The popliteal lymph nodes were excised 8 d later and cultured for 72 h in 96-well plates in supplemented RPMI 1640 at 5 × 10^5/well with the antigens indicated. Proliferative responses were assessed by the overnight incorporation of ^{125}I-deoxyuridine and results expressed as the mean cpm ± SEM of triplicate cultures.

Results and Discussion

Thyroiditis may be induced in high responder strains of mice by injecting Tg with a suitable adjuvant, usually CFA (11) or LPS (12), and in some studies, *Bordetella pertussis* has been used to augment the response (13). In our hands, thyroid lesions could be demonstrated in CBA/J mice on day 28 after the injection of 50 μg MTg/CFA in the base of the tail on day 0 and into the hind footpads on day 7. Equally effective was the injection of 50 μg MTg followed 3 h later by 20 μg LPS both administered intravenously and repeated on day 7.

In many reports, thyroiditis can be induced by adoptive transfer of spleen or draining lymph nodes from mice immunized with Tg and adjuvants (14–17), and we found that adoptive transfer produced better thyroiditis than in situ immunization. Draining lymph nodes or spleens taken 16 d after the first priming dose from animals immunized with MTg/CFA or MTg/LPS, respectively, were cultured in vitro for 3 d with antigen as described in Materials and Methods, before transfer to normal syngeneic recipients. Thyroids were examined 14 d later when lesions were nearly always observed as shown in Table 1, Exps. 3, 4, and 6.

We confirmed the observations of other groups (14, 17)

Table 1. *Transfer of Thyroiditis by T4 (2553) Peptide or MTg-primed Lymphocytes Reactivated In Vitro*

	Immunization		In vitro Stimulation		Cells injected			Highest score (day 14)						Average thyroiditis
Exp.	Antigen	Amount	Antigen	Amount	Cell	Amount	No. of mice	0	1	2	3	4	Incidence	± SEM
		μg		μg/ml										
1	MTg/CFA	50	pep	2.5	Lymph node	2 × 10^7	4			2*	2		4/4	1.69 ± 0.27
2	pep/CFA	5	pep	2.5			3			3			3/3	0.59 ± 0.17
3	MTg/CFA	50	MTg	40			3		1	2			2/3	0.63 ± 0.31
			pep	5			3				3		3/3	2.3 ± 0.1
	pep/CFA	20	MTg	40			2	2					0/2	0.04
			pep	5			3				3		3/3	2.73 ± 0.13
4	MTg/CFA	50	MTg	40			3			2	1		3/3	1.8 ± 0.61
	pep/CFA	20	MTg	40			2	2					0/2	0
			pep	5			3			1	2		3/3	2.14 ± 0.33
5	pep/LPS	20	MTg	40	Spleen	4 × 10^7	3	1			2		2/3	0.98 ± 0.5
			pep	5			3				3		3/3	3.25 ± 0.4
6	MTg/CFA	50	MTg	40	Lymph node	2 × 10^7	4			2	2		4/4	1.7 ± 0.075
			PPD	25			4	4					0/4	0.09 ± 0.02
			T4-free tryptic peptide	2.5			3	3					0/3	0.02 ± 0.03

* Number of animals with that grade of thryoiditis in one or more sections.

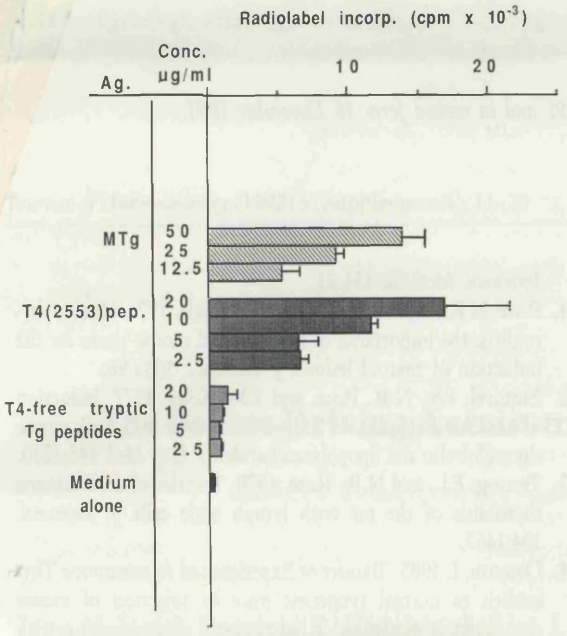

Figure 1. Proliferative response of MTg-primed cells to T4(2553) peptide in vitro. Popliteal lymph node cells from mice immunized in the footpad 8 d previously with MTg/CFA were stimulated for 72 h in vitro with MTg or peptide. Proliferative responses were assessed by the overnight incorporation of ^{125}I-deoxyuridine radiolabel and results expressed as mean cpm ± SE.

that in vitro activation with Tg antigens is essential for the transfer of thyroiditis to naive recipients (see Table 1, Exp. 6). Lymph node cells prepared from mice immunized with MTg/CFA 8 d earlier could be induced to proliferate by both MTg and the T4 (2553) peptide (Ac-STDD[T4]ASFSRAL-NH_2) (Fig. 1). This clearly shows that T cells recognizing the T4(2553) epitope, previously defined only by clonal T cell populations (6), are well represented in MTg-primed lymph node populations. To explore whether these T cells could elicit thyroiditis, the T4(2553) peptide was used to activate lymph node cells before transfer. MTg-primed lymph node cells cultured with T4(2553) peptide induced unequivocal thyroid lesions in all recipients examined (Table 1, Exp. 1).

In further experiments, mice were immunized with 5–20 µg T4(2553) peptide and either CFA or LPS as adjuvant. Lymph nodes or spleen were taken as before and activated for 72 h with peptide (2.5 or 5 µg/ml) before transfer. Every recipient of these cells had developed thyroid lesions by day 14 (Table 1, Exps. 2–5). These experiments show conclusively that the T4(2553) peptide itself can act as a thyroiditogenic epitope, since neither donor nor recipient was primed or challenged with whole Tg in an immunogenic form. Furthermore, the ability of the peptide to stimulate T but not relevant B cells (as judged by the absence of anti-MTg antibodies in the recipient) provides added confirmation of the importance of T cells as pathogenic mediators of the thyroiditis lesion.

In spite of repeated attempts to induce thyroiditis in situ with T4(2553) peptide and CFA or LPS at doses ranging from 1 to 25 µg, no convincing thyroid lesions have been seen. Furthermore, although draining lymph nodes from T4(2553) peptide/CFA-immunized animals could be stimulated in vitro with peptide to transfer excellent thyroiditis (as shown already in Table 1), stimulation of these same cells in vitro with MTg failed to produce any lesions in recipient animals (Table 1, Exps. 3 and 4). However, in one experiment, spleen cells from mice immunized with peptide and LPS, rather than CFA as adjuvant, did transfer thyroiditis (although there were fewer lesions) after activation in vitro with MTg (Table 1, Exp. 5). The reasons for these data are not clear at present. Experiments are in progress to investigate whether the choice of adjuvant affects the T cell subsets stimulated and whether LPS augments antigen presentation by triggered B cells, since it is quite possible that the concentration in vitro of the relevant peptide after processing of MTg by cells from T4(2553)/CFA-treated animals may not reach the threshold for effector activation (David Wraith, personal communication). Although it has been shown that the presence of B cells is not obligatory for the development of thyroiditis (18), they may well augment responses to certain determinants by more efficient presentation (19).

The ability of the T4(2553) peptide to induce thyroiditis under appropriate experimental conditions accords well with our previous studies on the importance of iodination for the activity of Tg in this system. However, our inability to date to induce thyroiditis directly by immunization with T4(2553) peptide/CFA in situ (under circumstances where MTg itself is effective) raises the possibility that other thyroxine-containing epitopes may be of pathogenic importance. The failure of Tg tryptic peptides depleted of T4 to boost MTg immunized cells in vitro (Table 1, Exp. 6) provides a further argument against the view that noniodinated epitopes play a role in the disease model although in fairness, it should be noted that the peptides were derived from human Tg and one cannot exclude the possibility that the noniodinated epitopes do not crossreact adequately with those derived from the mouse protein.

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The Forces Driving Autoimmune Disease

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There are two classes of autoimmune disease, organ-specific and non-organ specific or systemic. That cells producing autoantibodies are selected by antigen is strongly suggested by the presence of mutations and high affinity antibody. T-cells are pivotal in all forms of autoimmunity as evidenced by the therapeutic benefit of anti-T-cell monoclonals such as anti-CD4, and the frequent development of high affinity IgG autoantibodies. The production of anergic T-cells by the use of non-depleting anti-CD4 in the presence of antigen is discussed with particular reference to its potential for immunological intervention in autoimmune disease. It is possible to identify T-cell epitopes in organ-specific autoimmunity using pathogenic T-cell clones or hybridomas to identify the peptide sequences which are reactive. Antigen-specific therapy may ultimately be based on such peptide epitopes. The specificity of the T-cells in systemic autoimmunity is still obscure, but there is some evidence that reactivity with certain germ-line idiotypes can lead to the development of systemic autoimmunity. The possibility of stimulating B-cells specific for auto-antigens such as DNA becomes feasible if a complex of antibody and DNA is taken up by these specific B-cells and processed idiotype is presented to T-helpers specific for those idiotype epitopes.

Evidence is presented that there may be pre-existing defects in the target organ in certain organ-specific disorders, and the evidence for a glycosylation defect in the IgG in patients with rheumatoid arthritis is explored. It is noted that the spouses of probands with rheumatoid arthritis also tend to have this glycosylation defect and this raises the possibility of an effect due to an environmental factor, such as a microbial infection.

Molecular mimicry of autoantigens by microbes can stimulate auto-reactive cells by their cross-reactivity. It is emphasized that cross-reaction which gives rise to the priming of autoreactive T-cells could give rise to the establishment of a chronic autoimmune state. In animals with normal regulatory immune systems, such induced autoimmunity is ultimately corrected and it is only in animals where there are defects in regulation,

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that autoimmunity persists. Thus, there are many factors giving rise to autoimmunity, and the diseases are rightly regarded as multifactorial in origin.

Two classes of autoimmune disease

It is convenient to group autoimmune disease under two main headings, as first proposed by us many years ago [1]. In the organ-specific diseases the immune process is directed to antigens within a specific organ and as a result of aggressive action, leads to organ-specific lesions. Examples are Hashimoto's disease of the thyroid, pernicious anaemia affecting the stomach and myasthenia gravis involving autoimmunity to the acetylcholine receptors in the muscle endplate. In contrast, the non-organ specific diseases, involving a systemic autoimmunity, lead to the production of antibodies to autoantigens with a widespread organ and tissue distribution. There is, therefore, a more widespread location of the lesions, often considered to be representative of immune complex disease although this may be an over-simplified view. The rheumatological disorders, such as rheumatoid arthritis, SLE, scleroderma, dermatomyositis, Wegener's granulomatosis, etc., are the main contributors to this group of disorders. Fundamentally different mechanisms may underlie the two groups of diseases as evident from the fact that there is frequent overlap of diseases within each group but far less between the groups.

Currently, there are cogent advocates of the view that a pre-existing autoimmune state is intrinsic to the immune system and that a regulated network of lymphocytes linked through autoantigen and idiotype anti-idiotype interactions forms a part of the normal physiology of the immune system [2]. From this standpoint, failure to regulate this physiological autoimmune network leads to the emergence of pathogenic autoimmunity as evidenced by the appearance of destructive autoreactive T-cells and high affinity IgG autoantibodies.

B-cells producing autoantibodies are selected by antigen

This is not an entirely self-evident proposition since B-cells can be stimulated by polyclonal activators and by idiotypic interactions. Perhaps the most convincing evidence that B-cells are selected by antigen is the existence of somatic mutations [3] and high affinity in the autoantibody response. T-cell driven somatic mutation of B-lymphocytes can only lead consistently to a high affinity antibody response, if the appropriate B-cell mutants are selected by antigen itself in germinal centre structures. Additional support for the antigen-driven hypothesis comes from the occurrence of autoantibody responses to antigen clusters. The cluster may be represented by an intra-molecular linkage, as seen, for example, in the occurrence of several epitopes on the same autoantigenic molecule [4]. It is difficult to envisage a mechanism which could account for the co-existence of antibody responses to different epitopes on the same molecule, except through a linkage dependent on the structure of the antigen molecule itself. The same considerations apply to auto-

Short paper

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Active suppression induced by anti-CD4*

Treatment with a non-depleting monoclonal antibody to CD4 in the presence of mouse thyroglobulin (MTg) inhibits the development of murine autoimmune thyroiditis. This unresponsiveness was transferrable since such treatment generated a population of donor cells which could suppress the thyroiditis induced in lightly irradiated recipients by subsequent challenge with specific antigen. The suppression appears to be both antigen specific and antigen dependent and seems to discriminate between TH1 and TH2 helper subsets in that there is no significant effect on anti-MTg autoantibodies after challenge.

1 Introduction

Autoimmune thyroid disease affects up to 1% of the population and is characterized by the presence of infiltrating lymphocytes and circulating antibodies to thyroid antigens. The destructive lymphocytic population contains autoimmune T cells thought to escape from regulatory mechanisms and able to recognize self antigens in the gland [1, 2]. The exact chain of events which lead to this autoimmune state is not clear, although the thyroid antigens, thyroglobulin, thyroid peroxidase and the thyroid-stimulating hormone receptor have been characterized and much is now known about the epitopes responsible for activation of T cells [3, 4]. Any therapy which could selectively discriminate against the T cells making aberrant responses would greatly limit the widespread effects of general immunosuppression and leave legitimate responses intact.

Studies using a non-depleting (non-lytic) monoclonal antibody to the CD4 adhesion receptor on T cells have demonstrated selective long-term immunosuppressive effects in a number of experimental systems (*e.g.* responses to foreign proteins, marrow and skin grafts [5]; autoimmune diabetes [6] and murine lupus [7]) with the advantage that it is only those T cells recognizing antigen given under the antibody umbrella that become inactivated, while other responses remain intact.

It has been shown [5] that, in animals given antigen under cover of non-depleting anti-CD4, specific tolerance is induced, which can be maintained by repeated contact with antigen but without the need for further anti-CD4. If antigen is discontinued, however, animals eventually regain responsiveness, which is due to the influx of newly competent cells, since thymectomized animals remain unresponsive. As newly arising T cells are rendered unable to respond to repeated antigen injections, we must conclude that either they become tolerant as exquisitely susceptible "newborns" [8] or that they are kept from responding through coexistence with tolerant cells (Qin et al., in press). To test the hypothesis that the T cells in animals rendered tolerant by antibody therapy could actively suppress naive cells exposed to antigen, we used the murine autoimmune thyroid model [9] to adoptively transfer the putative suppressor population into naive recipients subsequently challenged with the antigen, mouse thyroglobulin (MTg).

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Abbreviations: **MTg:** Mouse thyroglobulin **EAT:** Experimental autoimmune thyroiditis

Key words: Autoimmunity / Thyroiditis / Suppression / Anti-CD4

2 Materials and methods

2.1 Preparation of antigens, mice, and immunization protocol

MTg was prepared by extraction from pooled homogenized thyroids (Parkes outbred strain reared in our own laboratory) as previously described [10]. CBA/J mice 8–12 week old, of either sex (NIMR, London, GB) were immunized in the base of the tail with 50 μg MTg emulsified in CFA (MTg/CFA; H37Ra; Difco Laboratories, Detroit, MI) and were boosted 7 days later with 25 μg MTg/CFA in both hind footpads. Where indicated, mice were exposed to 200 rad from a cobalt source either the day before transfer or earlier the same day.

2.2 Transfer of thyroiditis

The popliteal lymph nodes from MTg/CFA-immunized mice were taken on day 16 and cultured for 72 h in petri dishes at 5×10^6/ml in RPMI 1640 (Gibco, Paisley, Scotland) with 5% FCS and other supplements as described previously [4] together with MTg (40 μg/ml) to activate and expand effector T cells. After washing, 2×10^7 viable cells were injected intravenously (i.v.) into normal syngeneic recipients and thyroids were examined 14 days later.

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2.3 Evaluation of thyroid infiltration

Thyroids were fixed in 4% phosphate-buffered formalin and sections stained with hematoxylin-eosin. As described previously [9] for each mouse, there are 12 scores possible (two lobes at six levels): 0, no infiltration; 1, any definite infiltration up to 20%; 2, between 20 and 50% infiltration; 3, between 50 and 75% infiltration; 4, gland totally infiltrated but follicles still discernible; 5, no follicles detectable. The sections are read blind and the score for each mouse is the total divided by the number of observations (occasionally, one lobe may be missing, etc.). The average thyroiditis for each group is the mean ± SE of all the mice in the group.

2.4 Monoclonal antibodies to T cell antigens [11]

Depleting rat monoclonal antibodies to mouse cell surface antigens, L3T4 (YTS 191.1) and Ly-2 (YTS 169.4) (anti-CD4 and anti-CD8, respectively) and the non-depleting anti-CD4 monoclonal (YTS 177.1) were prepared as ascites in LOU × DA rats (Harlan OLAC Ltd.) pretreated with pristane. The ascites were ammonium sulfate precipitated and analyzed by SDS-PAGE for Ig content. The depleting antibodies were of the IgG2b, k subclass and were diluted in phosphate-buffered saline such that each mouse received 400-μg i.v. on the 1st day of treatment and 400 μg intraperitoneally (i.p.) on the 2 following days. The non-depleting anti-CD4 was subclass IgG2a, and 2 mg of this was given i.v., i.p. on consecutive days starting either the day before immunization with MTg or earlier on the same day and thereafter i.p. three times weekly continuing until after the antigen boost on day 7 (total of seven doses).

2.5 Enzyme-linked immunosorbent assay (ELISA)

Anti-thyroglobulin autoantibodies were assayed using standard ELISA methods [12] with a rabbit anti-mouse polyvalent Ig conjugated to alkaline phosphatase (Sigma Chemicals, Ltd.). The values shown are the antibody titers ± SEM required to give 50% of the max. control response.

2.6 Proliferation assay

Mice were immunized once in the hind footpad as described in the text and the popliteal lymph nodes were excised 7 days later and cultured for 72 h in 96-well plates in supplemented RPMI 1640 at 5×10^5/well with the antigens indicated. Proliferative responses were assessed by 6 h pulsing with ^{125}I-labeled deoxyuridine and results expressed as the mean cpm ± SEM of triplicate cultures.

3 Results

3.1 Non-depleting anti-CD4 abrogates primary responses to MTg

To ensure that the antibody could prevent a primary response *in vivo*, mice were immunized with MTg with and without anti-CD4 as detailed in the Sect. 2.1. Antibody responses were assessed on day 14 and on day 28, the thyroids were examined for infiltrating lymphocytes. Average thyroiditis for the untreated group was 2.13 ± 0.36 compared with 0.3 ± 0.18 in the group receiving anti-CD4 ($p < 0.002$). In the same mice, the anti-MTg antibody titer giving 50% of the maximum was reduced from 1100 to 160 (reciprocal).

3.2 Non-depleting anti-CD4 given to donor or recipient, prevents transfer of experimental autoimmune thyroiditis (EAT)

We and others have shown that thyroiditis can be transferred by spleen or lymph node cells from MTg/CFA-primed donors providing the cells are reactivated *in vitro* with the relevant antigen or with Con A before transfer [9, 13, 14]. When donor mice were treated with non-depleting anti-CD4 during the time of immunization as above, lymph node cells taken from these mice on day 16 and reactivated *in vitro* with MTg failed to transfer thyroiditis (Fig. 1, exp. 1, compare groups 1 and 2). Similarly, anti-CD4 given to recipients starting the day before transfer of activated lymph node cells, also prevented the development of thyroiditis (group 4). However, neither pulsing with anti-CD4 during the *in vitro* culture step (not shown) nor treating donors with antibody after immunization but 3 days prior to sacrifice (exp. 1, group 3) had any effect on the subsequent transfer of disease. In contrast, a *depleting* monoclonal anti-CD4 antibody given to donors 3 days before sacrifice, totally abrogated thyroiditis in recipient mice. In the same experiment, similar treatment with *depleting* anti-CD8 had no effect, thus, confirming the central role of $CD4^+$ T cells in the transfer of this disease (Fig. 1, exp. 2, groups 2 and 3). These experiments were repeated with similar results and suggested to us that the non-depleting anti-CD4-mediated non-responsiveness might not be simply a matter of receptor blockade (in which case, giving it to donors 3 days before sacrifice or adding it

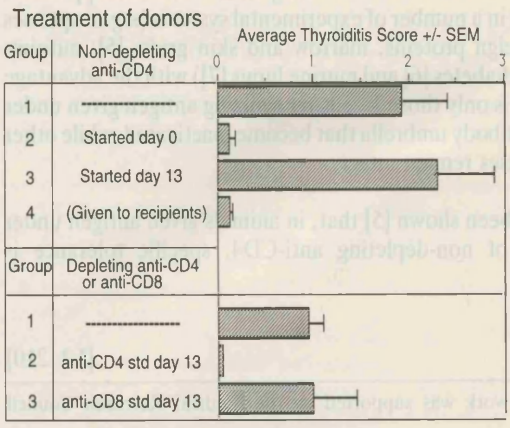

Figure 1. Transfer of thyroiditis abrogated by non-depleting anti-CD4. Depleting or non-depleting monoclonal antibodies were given to donor mice as indicated. Sixteen days after the first priming dose of MTg/CFA, the popliteal lymph node cells were reactivated *in vitro* with MTg 40 μg/ml for 72 h before transfer to normal syngeneic recipients (2×10^7 i.v.). In group 4, the non-depleting anti-CD4 was given to recipients instead, at time of transfer. Fourteen days after transfer, all recipient thyroids were assessed for thyroiditis (four mice group in exp. 1; three mice per group in exp. 2).

to the *in vitro* activation step should have been effective), but may have to do with a more permanent mechanism of down-regulation.

3.3 The anti-CD4-induced unresponsiveness is transferrable

To test the notion that cells made specifically unresponsive by treatment with anti-CD4 could actively suppress naive cells in the presence of antigen, the following experiments were carried out. Mice were immunized with MTg/CFA and anti-CD4 as previously. Three days after the second priming dose, lymph nodes were taken from donor animals and 2×10^7 cells were injected directly (*i.e.* after washing, but without an *in vitro* activation step) into syngeneic recipients. These recipients were lightly irradiated (200 rad) before transfer since this dose was shown by us to facilitate the transfer of tolerance, as induced by Kong et al., to high doses of soluble antigen [15, 16]. Control mice received cells from donors given PBS/CFA with or without anti-CD4. The recipients were challenged with 50 μg MTg/CFA on the day after transfer and again on day 7. Four weeks later, these mice were bled and thyroids examined for thyroiditis.

Fig. 2 shows a representative experiment demonstrating the suppressive effect exerted by donor cells on the recipient's subsequent response to antigenic challenge. In another experiment, thyroiditis scores of recipients given lymph node cells from donors primed with MTg/CFA + anti-CD4 were compared with those of recipients receiving cells from control donors primed with PBS/CFA + anti-CD4. The respective scores were 0.33 ± 0.08 and 2.1 ± 0.23 ($p < 0.002$). In a third experiment, recipients of lymph node cells from donors primed with MTg/CFA + anti-CD4 had an average thyroiditis score of 0.41 ± 0.21 compared with an average score of 2.14 ± 0.9 in mice which had been given 200 rad only.

3.4 Antibody responses in the recipient are unaffected by the suppressor cells which are specific for the tolerizing antigen

The 50% antibody titers shown in Fig. 2 are typical of all the experiments and show that the anti-MTg antibody response was not significantly affected in any of the groups. This makes it unlikely that the donor cells suppressed all aspects of the immune response. Furthermore, when putative suppressor lymph node cells were given to recipient mice challenged the next day with MTg, and the day after that with 2×10^8 SRBC i.p., there were no significant differences on day 5, in the numbers of Ig M and Ig G anti-SRBC plaque-forming cells per spleen [17], again indicating that the transferred cells were not generally immunosuppressive. To test specificity in a cell-mediated response, suppressor cells generated from mice immunized with MTg under cover of anti-CD4, were transferred to irradiated mice which were subsequently challenged with MTg/CFA or ovalbumin/CFA (OVA/CFA). Control mice did not receive suppressor cells. The proliferative response to MTg of MTg-primed lymph nodes from mice given suppressor cells was 2907 ± 136 *vs.* 6572 ± 902 in controls, whereas the response to OVA by OVA-primed lymph nodes

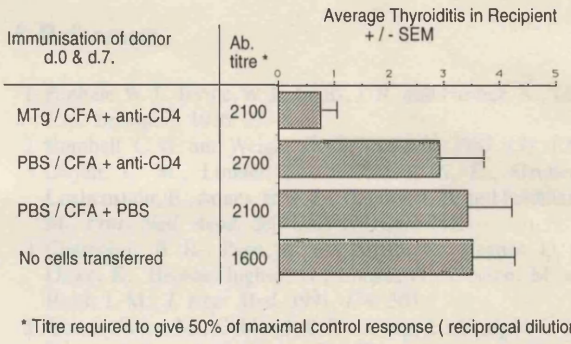

Figure 2. Active suppression induced by anti-CD4. The popliteal lymph nodes of donor mice immunized as shown, were excised on day 10 and after washing, 2×10^7 cells were transferred i.v. directly into recipient mice given 200 rad. These animals were challenged the following day in the base of the tail and 7 days later in the footpad, with 50 μg MTg/CFA. They were bled on day 14 and the thyroids were examined on day 28.

was slightly higher in the mice given suppressor cells than in the controls (4648 ± 904 *vs.* 3677 ± 863). The response to OVA was rather low, probably because conditions were not optimal for responses to this antigen, but clearly, there was no evidence of suppression.

4 Discussion

These experiments provide the most convincing evidence that something other than simple blockade and failure to prime occurs when MTg is given together with anti-CD4. Our data preclude the possibility of a direct effect of anti-CD4 in recipient animals, either by carry over, by permanent deletion of T cells or by down-regulation of the adjuvant effect of CFA, since lymph node cells from mice given anti-CD4 with PBS/CFA instead of MTg, were not suppressive. This observation also demonstrates the antigen-dependent nature of the active suppression and specificity was confirmed by the fact that responses to the irrelevant antigens SRBC and OVA, were not suppressed. Additionally, it has been shown, that the monoclonal antibody tolerizes to its own subclass as treated animals make no response to rat IgG2a but respond normally to rat IgG2b [5]. The findings in this report are wholly consistent with the observations of Qin et al. (in press) which indicate that maintenance of tolerance to an alloantigen established through anti-CD4/8 combination therapy, is achieved by a suppressor mechanism.

It has been suggested that T cells which recognize antigen while isolated from their sources of help become tolerant [18], and we think it probable that, in our experiments, naive cells do not receive appropriate signals when they bind to processed antigen on the antigen-presenting cell in the presence of large numbers of unresponsive cells. This is unlikely to be due simply to overcrowding or competition for resources since lymph node cells from anti-CD4-treated but unprimed animals, do not have the same effect.

One interpretation of our data would be that confrontation with antigen in the presence of anti-CD4 has a differential effect on TH1 and TH2 cells, such that TH1 are permanently modified, whereas TH2 are only temporarily

blocked. If this were the case, both thyroiditis and autoantibody production would be abrogated in animals treated directly with the antibody. On transfer, however, TH2 cells emerging from their anti-CD4 block, could lead to an overall predominance of TH2 cells relevant to TH1 in recipient mice. Cytokines secreted by TH2 cells *e.g.* IL-4, IL-10 and TGFβ, are known to down-regulate TH1 cells [19, 20, 21] and this could mean that naive TH1 cells in recipients would be unable to respond to antigenic challenge. However, since TH2 cells would not be affected, the autoantibody response would be normal. We have not formally identified the active cell as a T cell, although the fact that it is induced by a monoclonal antibody specific for the CD4 receptor suggests that it is. A preliminary experiment indicated that $CD8^+$ cells were also involved in the final effect since treatment of recipient mice with a depleting antibody to CD8 abrogated the suppression.

Whatever the mechanism, we conclude that priming under cover of anti-CD4 generates a population of regulatory cells capable of actively and specifically suppressing the response of a naive population not exposed to circulating anti-CD4. Whether this effect is transitory and mediated, as already suggested, by locally produced cytokines, or whether the naive cells are permanently anergized, remains to be established. Preliminary experiments (manuscript in preparation), in which the putative tolerizing lymph node cells were mixed with MTg-primed, potentially thyroiditogenic effector cells *in vitro*, have shown that even primed cells can be "switched off" and indicate a possible role for such potent regulatory cells in the management of aberrant or undesirable responses.

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