T cells and thyroid autoimmunity

ABSTRACT—Autoimmune thyroid disease is the archetype of organ-specific autoimmune disorders and shares with them T cell dependence. The observation that thyroid cells in autoimmune thyroid disease express the major histocompatibility complex molecule HLA-DR led to the hypothesis that they could present antigen and initiate or maintain the autoimmune process. However, functional experiments, and recent evidence indicating that provision of a co-stimulatory signal is also essential for efficient antigen presentation, argue against such a role. The analysis of T cell responses to two major thyroid antigens, thyroid peroxidase and the thyroid stimulating hormone receptor, reveals a heterogeneity both within and between patients, and intrathyroidal T cells show diverse usage of T cell receptor genes. Therefore, any strategy that uses modified peptides, monoclonal antibodies against specific T cell receptor molecules, or T cell vaccination for the purpose of treating thyroid autoimmunity is unlikely to succeed.

The immune system probably evolved to discriminate infectious non-self from non-infectious self [1]. In normal unimmunised animals, it generates natural antibodies which can react with a variety of self constituents and exert a range of biological actions, some unrelated to immunity [2]. There are multiple immunoregulatory networks for both B and T cells which maintain self tolerance despite the simultaneous persistence of autoreactive cells. A failure of these controlling networks permits the transition from autoreactivity to autoimmune disease. In this review, recent developments in the understanding of autoreactive T cells are discussed and in particular how these may be controlled therapeutically. The involvement of T cells in autoimmune diseases affecting the thyroid is then considered.

T cell tolerance

The processes leading to discrimination between self and non-self by T cells include central tolerance (induced in the thymus), peripheral tolerance, and active suppression. T cells are stimulated when the T cell receptor (TCR) recognises the bimolecular complex of processed antigen and the major histocompatibility complex (MHC) presented to them by antigen presenting cells (APC) such as macrophages and dendritic cells. The recognition of the complex of self-peptide with self-MHC during ontogeny results in the deletion of most potentially autoreactive T cells, first shown for those expressing the Vβ17a variable region segment of the TCR, which are deleted in the thymus of mice expressing the I-E MHC molecule [3]. This observation was complemented by experiments involving transgenic mice whose cytotoxic T cells preferentially recognised the male transplantation antigen, H–Y. In female animals, about 15% of the peripheral $CD8^+$ T cells expressed the transgenic receptor for H–Y, whereas such T cells were extremely rare in male mice [4]. In male mice constitutively expressing H–Y, the cytotoxic transgenic cells responding to H–Y were largely deleted in the thymus, although a few escaped and were detectable in the periphery.

From these and many similar observations, it is apparent that additional mechanisms are required to regulate the autoreactive cells escaping deletion. One mechanism is clonal anergy, which has been shown directly in mice expressing minor lymphocyte-stimulating (Mls) antigens that interact with particular TCR Vβ receptor gene segments. For example, T cells expressing receptors of the Vβ6 and Vβ8.1 families interact with $Mls–1^a$ antigen whereas those expressing receptors of the Vβ3 family respond to the $Mls–2^a$ antigen. Immunising $Mls–1^b$ mice with $Mls–1^a$ expressing cells induces anergy in the $V\beta6^+$ T cells of the recipient. These cells are still present but are non-reactive [5]. The mechanism for anergy induction remains to be elucidated.

Two broad extrathymic mechanisms deal with autoreactive T cells escaping central tolerance: peripheral tolerance and active suppression (Fig 1). Clonal expansion of all lymphocytes requires both receptor ligation and the simultaneous receipt of poorly defined co-stimulatory signals [6]. Early work indicated that treatment of APC with ultraviolet light or heat inactivation could destroy the immunogenicity of the APC without affecting their antigenicity [7]. These authors referred to the lost activity as 'co-stimulatory'. In its absence, antigen presented by an MHC class II molecule may have no effect or may result in T cell anergy.

This latter phenomenon is termed peripheral tolerance, and is best exemplified again in transgenic mice whose pancreatic β cells alone express the I–E class II molecule. These mice fail to delete their $V\beta17a^+$ T cells and do not develop insulitis [8]. More importantly, the $I–E^+$ β cells induce specific T cell tolerance because they are unable to provide a co-stimulatory

N TANDON, MD
Cambridge Nehru Scholar
A P WEETMAN MD, FRCP
Professor of Medicine
Department of Medicine, University of Sheffield
Clinical Sciences Centre

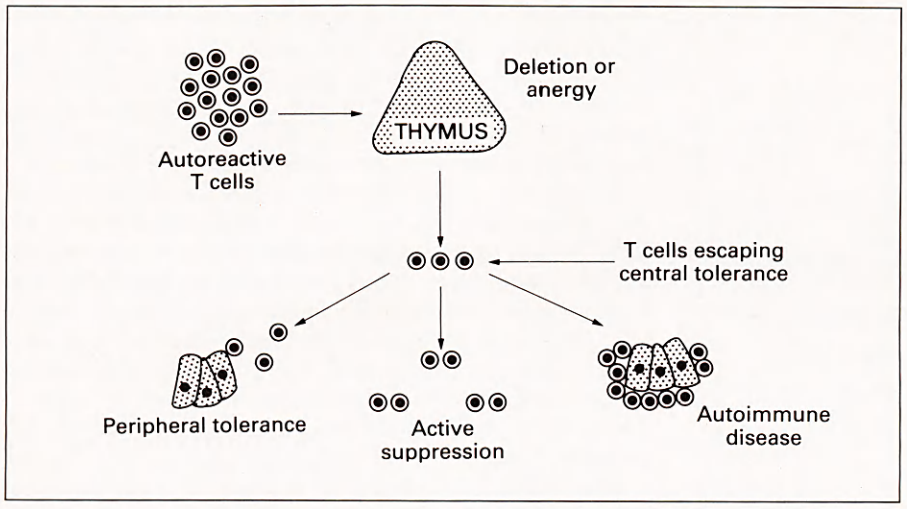

Fig 1 *Extrathymic mechanisms of tolerance: peripheral tolerance and active suppression.*

signal [9]. Peripheral tolerance has also been demonstrated in many other experimental systems, including hapten-modified mouse splenocytes and human class II$^+$ keratinocytes [10,11]. Peripheral tolerance to transgenic class I MHC molecules expressed by β cells also occurs, but can be reversed *in vitro* by providing optimal amounts of antigen interleukin–2 (IL–2) [12]. Thus, if cytotoxic T cells which have escaped central tolerance are exposed to the relevant antigen in the presence of IL–2 (provided by untolerised CD4$^+$ T cells), autoimmune injury may be initiated. This has been confirmed in recent experiments with transgenic mice, indicating that autoreactive T cells may cause autoimmune disease when provided with exogenous 'help' in the form of IL–2 [13].

Two other mechanisms have been implicated in peripheral tolerance [14].

1. High avidity interaction between T cells and APC may lead to clonal deletion, intermediate avidity may lead to anergy, and low avidity may not influence T cell behaviour at all. However, reliable measures of TCR affinity to test this have only just been established.
2. Persistence of antigen under immunogenic conditions may lead mature T cells towards exhaustive differentiation and clonal elimination. Also, persistent antigen may anergise T cells which have recently emigrated from the thymus (these cells are more sensitive to tolerogenesis).

Active suppression also participates in controlling autoreactive T cells, but remains controversial. Dorf and Benacerraf [15] have described in detail the various levels of CD8$^+$ T cell-mediated immunoregulation that suppress delayed-type hypersensitivity in the mouse. On the other hand, some have questioned the very existence of suppressor cells, as these have not been isolated and adequately characterised [16]. However, antigen-specific CD8$^+$ suppressor cell clones have now been established from patients with leprosy which can inhibit CD4$^+$ T cell proliferation induced by *Mycobacterium leprae*. These suppressor cells recognise specific antigen in an MHC-restricted fashion, but their effect is not specific or MHC-restricted. They induce inactivation or anergy in the CD4$^+$ population by an unknown mechanism [17]. Cytokine release may be involved: IL–4 produced by stimulated CD8$^+$ T cell clones (designated type 2 CD8$^+$ T cells) is necessary for suppression of proliferation of IL–2 and γ–interferon(IFN)-secreting T cell clones [18]. Additional explanations for T suppressor cell effects include:

- release of antigen-specific suppressor factors [19];
- cytotoxicity to helper cells [20]; and
- reactivity of the suppressor cells against the variable region (idiotype) of the TCR on the target clone, thus behaving as anti-idiotypic cells [21].

The T cell receptor in autoimmune disease

The TCR-MHC interaction requires both processing of antigen and its presentation by an MHC molecule (Fig 2). Polymorphic residues around the MHC peptide cleft determine the ability of allelic MHC molecules to bind specific peptides, and this peptide –MHC complex is recognised by specific TCRs. The TCR of mature T lymphocytes comprises two highly variable glycoproteins (either an αβ or γδ pair) noncovalently associated with five other invariant molecules constituting the CD3 complex. The αβ complex is present on over 95% of peripheral blood T cells, the remainder expressing the γδ TCR. TCR proteins are similar to immunoglobulin molecules, comprising variable (V), diversity (D), joining (J) and constant (C) regions, with the highly variable membrane distal domains being responsible for antigen binding. The gene segments encoding these regions recombine in various combinations in the developing T cell to produce a continuous exon. The large number of gene segments permits many transcriptional permutations, increasing TCR diversity and enabling the recep-

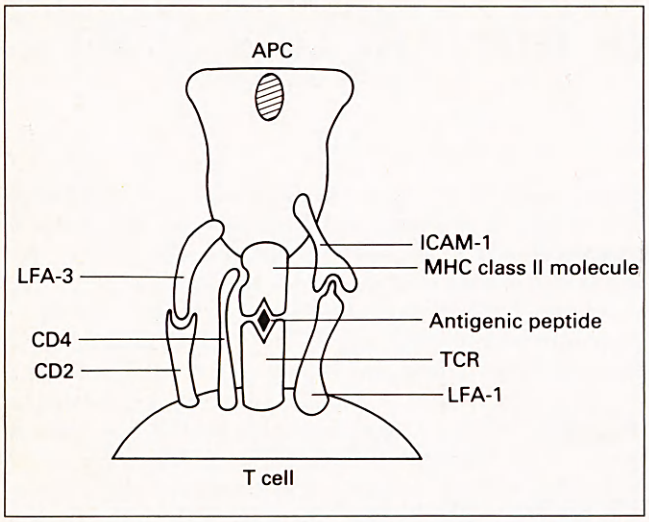

Fig 2 *Antigen presentation: major adhesion molecules involved in APC-CD4⁺ T cell interaction.* The antigenic peptide is recognised by the TCR in the context of the MHC molecule. This interaction is strengthened by adhesion molecules. (APC = antigen presenting cell; ICAM = intercellular adhesion molecule; LFA = lymphocyte function-associated antigen; MHC = major histocompatibility complex; TCR = T cell receptor).

tors to recognise a vast number of antigens. There are approximately 50 Vα and 57 Vβ gene segments in the human genome.

Little information is available on the human Vα repertoire, but Moss *et al* [22] report a broadly similar profile in different individuals, with threefold differences seen for some Vα segments such as Vα4 and Vα11. The repertoire is indistinguishable between identical twins, suggesting a predominant genetic influence on the Vα usage. Similarly, no major variations have been reported in the human Vβ repertoire. Different individuals of random HLA type have a broadly similar profile of Vβ repertoire, though a genetic component is involved, as shown by the similarity of Vβ usage in identical twins [23].

Experimental allergic encephalomyelitis (EAE) is an animal model of multiple sclerosis in which autoaggressive T cells may express a limited set of TCRs. Molecular evidence for a shared element was provided by studies showing common rearrangements of TCR β chains in EAE-inducing T cell clones from the Lewis rat [24]. Analysis of messenger RNA (mRNA) from encephalitogenic Lewis rat T cells demonstrated that these TCRs express the same Vα and/or Vβ regions [25]. Further work demonstrated that the majority of encephalitogenic T cell clones from PL/J mice express the Vβ8 region [26], and that treatment with Vβ8 monoclonal antibodies prevents disease [26,27]. This paved the way for evaluating the possibility of targeting the disease-related TCR for immunotherapy. For instance, vaccination with synthetic peptides corresponding to idiotypic determinants of the hypervariable region of the Vβ8 TCR protects rats against EAE [28,29], and a synthetic peptide corresponding to residues 39–59 of Vβ8 not only protects animals from subsequent induction of EAE but also ameliorates disease when given to animals with established EAE [30].

In general, however, TCR usage in autoimmune models may not be so limited [31], and the role of MHC-binding peptides in the immunotherapy of autoimmune disease has also been assessed. Myelin basic protein (MBP) is the major autoantigen in EAE, and peptide analogues of its N-terminal 1–9 peptide were shown by Wraith *et al* [32] to inhibit the induction of EAE. This work has been confirmed by others: competitive peptides [33,34] and soluble MHC class II peptide complexes [35] both inhibit EAE.

These data have prompted analysis of T cell populations responsible for autoimmune disease in humans, concentrating on T cells recovered from the site of autoimmune inflammation and on those that are responsive to candidate autoantigens *in vitro*. But results have been difficult to interpret (Table 1). In some of the earliest studies, T cells cloned from the cerebrospinal fluid of multiple sclerosis patients had a limited number of TCR β chain rearrangements [36], and only a restricted number of Vα families were used by T cells in plaque lesions [37]. However, unlike animal studies, the pattern of Vα usage varied considerably from patient to patient. Moreover, restricted V-region usage has been reported in tissue-infiltrating T cells from non-autoimmune disorders like intraocular melanomas [38] and cutaneous T cell lymphomas [39], as well as from various sites in normal individuals.

Even more uncertainty resides in the analysis of V-region usage by T cells that react with potential autoantigens *in vitro*. Reported Vβ restriction of T cell lines responsive to human MBP [40] contrasts with another study in which 17 clones from a single patient, responding to the same determinant on this autoantigen, showed 12 different β chain rearrangement patterns [41]. Although clones responding to MBP may show a bias towards Vβ5.2 usage, it is not equally strong in all patients [42, 43]. This heterogeneity is likely to be compounded by the involvement of other autoantigens in multiple sclerosis. Although EAE and multiple sclerosis have served as paradigms in these developments, many autoimmune disorders have been similarly investigated, including those of the thyroid discussed below.

T cell-thyrocyte interactions

Adhesion molecules

Cell-cell interactions are mediated through adhesion molecules which influence not only adhesion (Fig 2) but also cellular growth, differentiation, junction formation and polarity. These molecules can be grouped as:

Table 1. Problems associated with interpreting T cell receptor restriction in human autoimmune diseases

Technique	Comment
Monoclonal antibodies to stain Vα or Vβ families	Direct and quantitative; inadequate range of monoclonal antibodies at present.
	Non-autoreactive T cells included.
PCR amplification of Vα or Vβ mRNA directly from affected tissues.	Semi-quantitative at best. Total T cell population examined; some irrelevant (eg blood-borne) T cells will be included.
	In chronic disease, the population may poorly reflect events at initiation.
Selection of putative, autoreactive T cells (eg IL-2 receptor-positive) before PCR.	May introduce bias.
Cloning of autoreactive T cells before PCR.	Difficult to establish human clones. Need to know autoantigens. *In vitro* culture may introduce bias.

IL = interleukin
mRNA = messenger RNA
PCR = polymerase chain reaction

- integrins, heterodimeric molecules functioning as cell-cell and cell-substratum receptors;
- molecules of the immunoglobulin superfamily, involved in cell–cell adhesion and important in inflammation;
- cadherins, calcium-dependent homophilic cell–cell adhesion proteins;
- cell adhesion molecules with lectin-like domains (LEC-CAMs) mediating white blood cell/endothelial cell adhesion; and
- homing receptors that direct lymphocytes to specific lymphoid tissue [44].

The adhesion between T cells and their targets is a fundamental component of any immune response, and consequently modulation of the expression of adhesion molecules by target cells will have important consequences for autoimmune disease [45].

Thyroid follicular cells (TFC) in autoimmune thyroid disease express intercellular adhesion molecule-1 (ICAM–1), the ligand for lymphocyte function-associated antigen-1 (LFA–1) present on T cells [46]. The expression of ICAM–1 can be enhanced *in vitro* by the cytokines γIFN, IL–1 and tumour necrosis factor (TNF), and thus seems likely to depend on release of these cytokines by the infiltrating T cells. The functional relevance of ICAM–1 expression on human TFC can be demonstrated by the reduction in lymphocyte-TFC cluster formation with a blocking ICAM–1 monoclonal antibody [47].

These findings have been confirmed in Hashimoto's thyroiditis [48,49], but there are conflicting data regarding the expression of ICAM–1 on TFC in Graves' disease. Some reports indicate that ICAM–1 can be found in these cells [47,50,51], but others do not confirm these findings [49,52]. However, Northern blot analysis and *in situ* hybridisation have revealed ICAM–1 mRNA in TFC from patients with Graves' disease [53]. TFC also express LFA–3 (CD58) which binds to CD2 on T cells [48]. Like ICAM–1, LFA–3 is upregulated by cytokines and participates in T cell adherence to TFC, but ICAM–2, another ligand for LFA–1, is not expressed on TFC [54]. These observations show that T cell binding to TFC targets is likely to be facilitated in autoimmune thyroiditis through enhanced adhesion molecule expression.

Antigen presentation by thyroid follicular cells

The observation that TFC also express MHC class II molecules in Graves' disease and Hashimoto's thyroiditis led to the hypothesis that they could become APC, capable of stimulating T cells by presenting endogenous autoantigen [55]. However, aberrant class II expression is unlikely to be the initiating event in autoimmune thyroiditis, as it depends on local T cell infiltration. Three lines of evidence support this premise. First, the only clearly defined inducer of class II expression is γIFN, and TFC from patients with or without autoimmune thyroid disease express class II molecules equally well *in vitro* when cultured with γIFN [56, 57]. Secondly, the distribution of class II^+ TFC in autoimmune thyroid disease correlates with the presence of γIFN-containing lymphocytes, implying a direct relationship between the two [58]. Thirdly, in

time course studies of experimental autoimmune thyroiditis in rats, the lymphocytic infiltrate always appears before class II molecules on TFC [59].

Thus it seems more likely that TFC class II expression occurs secondarily to the lymphocytic infiltration than that it initiates the autoimmune process. Whether class II^+ TFC can perpetuate the immune response remains equivocal. Primary cultures of class II^+ TFC present influenza peptides to histocompatible peptide-specific T cell clones and can stimulate T cells in the autologous mixed lymphocyte reaction [60,61]. In other experiments, T cell lines or clones, expanded from the intrathyroidal lymphocytes by culture with IL–2, proliferated weakly in response to autologous class II^+ TFC, suggesting that they have an antigen-presenting role [62–64]. However, class II^+ TFC have been found only weakly and inconsistently to stimulate allogeneic peripheral blood T cells [65]. Moreover, primary cultures of mouse TFC, stimulated with γIFN to induce class II molecules, failed to present thyroglobulin, foreign antigen or alloantigens to T cells [66,67].

These variable results could be explained by contamination of the primary TFC cultures with a small proportion of mononuclear cells. It is the release of γIFN by these cells that induces class II expression when TFC are cultured with T cell mitogens [57]. The antigen-presenting potency of dendritic cells, in particular, is probably sufficient to allow small numbers to produce the observed effects, and the increased intrathyroidal number of these 'professional' APC in Graves' disease and Hashimoto's thyroiditis [68] makes this more likely. Certain rat and mouse thyroid-derived epithelial lines can present antigen, although an additional stimulus (phorbol ester) was required for one of them [69,70]. Furthermore, the process of establishing cell lines may radically alter cell behaviour, so that these observations may have little relevance to disease pathogenesis.

Failure to deliver a co-stimulatory signal is the likely explanation for the inability of TFC to act as efficient APC. Early interest focused on γIFN and IL–1 as co-stimulators, but γIFN is not produced by endocrine cells and evidence for the synthesis of IL–1 by TFC remains conflicting [71,72]. As shown in Fig 3, treatment of TFC with phorbol esters, which activate protein kinase C, results in a consistent enhancement of the ability of primary TFC cultures to stimulate T cell alloreactivity [65]. That suggests that, *in vitro*, phorbol esters can induce the production of a co-stimulator missing under normal circumstances, but the relevance of this to autoimmune thyroid disease remains to be established.

Two other molecules have been identified as co-stimulators: B7 [73] and the heat-stable antigen [74]. B7 is a B cell activation marker which has also been identified on γIFN-treated monocytes [75], dendritic cells [76], and even non-haematopoietic cells like keratinocytes [77], but using immunohistochemistry and flow cytometry, it has not been possible to demonstrate the presence of this molecule on TFC (unpublished data). Less is known about the heat-stable antigen though B7 and heat-stable antigen cooperate in co-stimulating murine CD4 T cell growth [74].

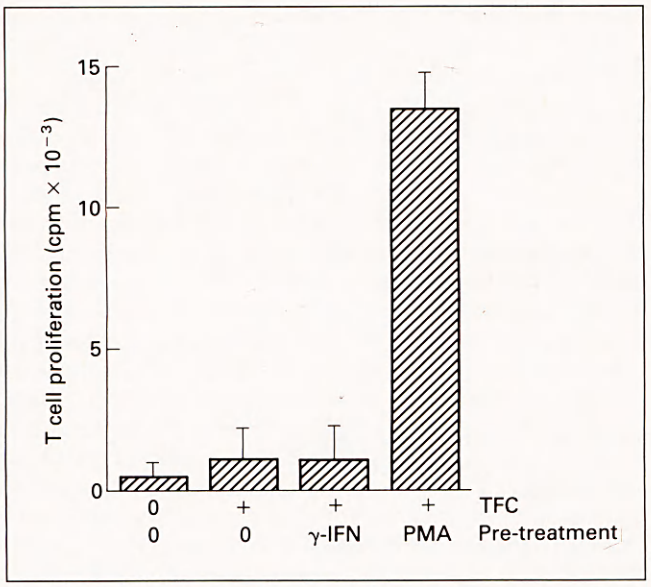

Fig 3 *Effect of γ-interferon and phorbol myristate acetate treatment on the ability of thyroid follicular cells to stimulate T cells, shown as proliferation.* The results are shown as mean + SD. (IFN = interferon; PMA = phorbol myristate acetate; TFC = thyroid follicular cells)

In summary, class II expression by TFC is believed to be unlikely to initiate or even perpetuate thyroid autoimmunity. As the intrathyroidal infiltrate contains abundant dendritic cells and B cells, both of which can act as APC for thyroid autoantigens [78], it is doubtful whether any additional antigen-presenting capacity provided by class II^+ TFC would have a significant impact on disease evolution. It is unclear at present whether TFC are capable of supplying the necessary second signal *in vivo*, even though it can be induced *in vitro*. In its absence, it is possible that TFC class II expression may operate to induce peripheral tolerance. This would explain why autoreactive T cells have been so difficult to culture from Graves' disease patients.

T cell responses to thyroid antigens

The possibility of using modified synthetic peptides to treat autoimmune disease has prompted the search for immunodominant epitopes on putative thyroid autoantigens. The pathogenicity of the immune response mounted against thyroglobulin is still open to debate, so most studies have evaluated the T cell responses to thyroid peroxidase (TPO) and the TSH receptor (TSH-R). In particular, safe and specific immunotherapy for Graves' disease would be a consid-

erable improvement over current treatment which often results in gland destruction.

T cell responses to thyroid peroxidase

TPO has been identified as the thyroid microsomal antigen [79]. T cell responses to it may be of critical importance in the pathogenesis of autoimmune thyroid disease, as thyroiditis in mice can be induced with TPO-specific T cells independent of TPO antibody formation [80]. Fukuma *et al* [81] reported weak T cell proliferative responses in Hashimoto patients to a random selection of 12 TPO peptides predicted, by a computer algorithm, to contain T cell epitopes. The T cell proliferative responses to a panel of 16 synthetic peptides (representing 23% of the total sequence), also predicted to contain T cell epitopes, were further evaluated but no single peptide consistently elicited a response from lymphocytes of patients with autoimmune thyroid disease [82]. Three separate T cell epitopes in amino acid residues 415–432, 439–457 and 463–481 were identified to which T cells from between 23% and 27% of patients responded. It is reasonable to expect more T cell epitopes to be located in other regions of the molecule.

Ewins *et al* [83] analysed eight discrete recombinant fragments encompassing the whole of the extracellular region of the TPO molecule. They found significant Hashimoto T cell proliferation in response to the full length affinity-bound TPO molecule as well as to the recombinant fragments R1c (residues 145–250) and R2b (residues 457–589). Region R2b encompasses the sequence 463–481 noted above. Using T cell lines and clones derived from a single Graves' patient, two of a series of TPO synthetic peptides (residues 535–551 and 632–645) stimulated proliferation [84].

T cell responses to the thyroid-stimulating hormone receptor

The TSH-R is the receptor in the thyroid for stimulation of thyroid growth and hormonogenesis. Although many potential binding sites for thyroid stimulating antibodies have been identified [85,86], information on T cell responses to TSH-R is meagre. T cell proliferation was assessed using 28 peptides spanning the entire extracellular region of the molecule. There was no difference between controls and patients with Graves' disease [87]. The T cells from the responding patients reacted to varying numbers of peptides, two patients responding to as many as nine peptides, and depletion of the $CD8^+$ population (putative suppressor cells) did not affect the response. Akuso *et al.* [88] assessed responses to synthetic peptides representing different, randomly selected segments of the extracellular domain (pool A; 3 peptides) and extracellular loops of the membrane spanning region (pool B; 3 peptides). Two of their hyperthyroid patients responded to pool A, and this number increased to seven after $CD8^+$ T cell depletion. However, the limitations of this study include variability in the size of the peptides, random distribution of the regions under study and the absence of any control peptide in the assays.

Although T cell epitopes and immunogenic regions have been identified on the TPO and TSH-R molecules, different patients respond to different epitopes and the same patient may respond to multiple sequences. These observations are not surprising, given the long evolution of autoimmune thyroid disease in contrast to acutely induced experimental models. Even in EAE, determinants of MBP which are not recognised after primary immunisation can become immunogenic in the course of the disease [89]. This diversification of the autoreactive T cell repertoire to include recognition of cryptic determinants has been termed 'spreading', and poses a major limitation for novel therapeutic approaches to T cell-mediated autoimmune disease. These facts make it unlikely that intervention with modified peptides would be a viable therapeutic option to block the immune response to thyroid antigens.

Restriction of the T cell repertoire

Restricted usage of TCR V genes by autoreactive T cells could lead to immunointervention specifically targeting the TCR of the cells involved in disease causation. However, using monoclonal antibodies against three TCR Vβ families Teng *et al* [90] found no abnormal distribution in Graves' intrathyroidal lymphocytes; also restriction fragment length polymorphism (RFLP) analysis of TCR gene rearrangements in intrathyroidal T cells from patients with Hashimoto's thyroiditis provided evidence suggestive of clonal expansion in only one of six patients [91,92]. A similar study in patients with Graves' disease also suggested a polyclonal expansion [93].

An alternative approach has been to use the polymerase chain reaction to amplify V gene segment-specific reverse-transcribed mRNA, although quantitation is difficult with this technique. Davies *et al* [94] reported that Vα gene usage by intrathyroidal T cells from patients with autoimmune thyroid disease was restricted in comparison with matching peripheral blood lymphocytes, but the restricted Vα genes differed between patients, with no association with the type of disease. There was no restriction of Vβ usage [95]. In a further study, Davies *et al* [96] examined the Vα and Vβ usage of intrathyroidal T cells present in the aspiration thyroid biopsies. They demonstrated significant Vα and Vβ gene restriction in Graves' disease but much less restriction of both families in Hashimoto's disease. In contrast, it has not been possible to show restriction of intrathyroidal TCR Vα families in other patients with Graves' disease [97]. Even examining IL-2 receptor-positive intrathyroidal lymphocytes (ie activated T cells), and microheterogeneity within particular Vα families, there was little evidence for restricted Vα usage relative to peripheral blood [98].

From these conflicting data it is difficult to resolve whether any restriction exists in the T cell repertoire in autoimmune thyroid disease by the time of diagnosis, although this is believed likely, at best, to be only quantitative rather than qualitative. Even when shown to exist there has been no consistent bias in favour of any one Vα or Vβ family. This again casts doubt on the feasibility of an immunotherapeutic approach based on targeting a specific TCR in thyroid autoimmunity.

Although these results are somewhat disappointing for prospects of immunotherapy, they have none the less provided useful insights into the pathogenesis of Graves' disease and Hashimoto's thyroiditis. The accumulating evidence that cytokines have a major role in thyroid and other endocrine autoimmune disorders has been only briefly considered in this review. It is possible that therapeutic strategies to reduce cytokine activity could be successful in these conditions. This seems the most promising direction for the next decade.

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Address for correspondence: Professor A P Weetman, Department of Medicine, University of Sheffield Clinical Sciences Centre, Northern General Hospital, Sheffield S5 7AU.

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