Clin. exp. Immunol. (1990) **82,** 275–283

Thyroid autoantigens and human T cell responses

N. FUKUMA*, S. M. MCLACHLAN*, B. RAPOPORT§, J. GOODACRE†, S. L. MIDDLETON†, D. I. W. PHILLIPS*, C. A. S. PEGG‡ & B. REES SMITH* *Endocrine Immunology Unit, Department of Medicine, University of Wales College of Medicine, Cardiff, †Department of Medicine (Rheumatology), University of Newcastle upon Tyne, Newcastle upon Tyne, ‡Department of Surgery, University of Nottingham, Nottingham, UK and §Department of Medicine, University of California, San Francisco, CA, USA

(Accepted for publication 21 June 1990)

SUMMARY

We investigated the ability of T cells from patients with Hashimoto's thyroiditis and with Graves' disease as well as control donors to proliferate in response to thyroid peroxidase (TPO) and thyroglobulin using (i) lymphoid cells from different lymphoid organs; (ii) unfractionated or CD8-depleted lymphoid suspensions or T cells + autologous low density cells (LDC); (iii) 200-μl well cultures and 20-μl hanging-drop microcultures; and (iv) intact TPO and thyroglobulin, denatured thyroglobulin and 12 synthetic peptides predicted on the basis of the amino acid sequence of TPO to be T cell epitopes. In 200-μl well cultures, proliferative responses (assessed in terms of ^{3}H-thymidine uptake) to intact TPO or thyroglobulin, digested thyroglobulin or synthetic TPO peptides were not significantly different in unfractionated or CD8-depleted lymphoid suspensions from blood, thyroid or lymph nodes of TPO/thyroglobulin autoantibody-positive patients, autoantibody-negative patients or control donors. In contrast, blood T cells from some high titre patients with Hashimoto's thyroiditis (but not from healthy individuals) proliferated in response to intact thyroglobulin or TPO presented by autologous LDC in hanging-drop microcultures. Heat denatured thyroglobulin (with which thyroglobulin autoantibodies do not interact) did not stimulate proliferation and this observation, together with the ability of T cells from some patients to respond to intact thyroglobulin in the absence of LDC, indicated that thyroglobulin-specific B cells may be involved in antigen presentation. As we were unable to demonstrate proliferation by blood T cells + LDC from all thyroglobulin antibody-positive patients with Hashimoto's thyroiditis, our studies suggest that the presence of sufficient precursor T cells, as well as the number and type of antigen-presenting cells, are critical for T cell proliferative responses to human TPO and thyroglobulin.

Keywords dendritic cells peptides proliferation thyroglobulin thyroid peroxidase

INTRODUCTION

The processes involved in thyroid epithelial cell destruction in Hashimoto's thyroiditis are complex and probably include damage by cytotoxic T cells; by autoantibodies to thyroid peroxidase (TPO) and thyroglobulin by activation of complement and/or antibody dependent cell cytoxicity; and by the adverse effects of cytokines such as interferon-gamma (IFN-γ) and interleukin-1 (IL-1). T cells play a crucial role in thyroid damage since in addition to direct cytotoxic effects (Kong, Bagnasco & Canonica, 1986; MacKenzie *et al.*, 1987), they produce cytokines like IFN-γ and provide 'help' for B cells capable of secreting autoantibodies to thyroglobulin and TPO (Forouhi *et al.*, 1987). Consequently, a detailed analysis of T cell responses to thyroid antigens such as TPO and thyroglobulin necessary to understand fully human autoimmune thyroid destruction.

Substantial evidence suggests that T cells recognize short, linear fragments of protein antigens that bind to a cleft in MHC class I and class II molecules on antigen-presenting cells (Bjorkman *et al.*, 1987). A variety of bone-marrow-derived cells is involved in antigen presentation, including dendritic cells (Steinman *et al.*, 1979) which are potent antigen-presenting cells for several antigens. Some antigens may need to be 'processed' to provide suitable fragments for presentation (reviewed by Allen, 1987). Synthetic peptides have been used to activate T cells and several studies have suggested that it may be possible to predict the sequence of amino acids likely to form T cell epitopes in a protein antigen (De Lisi & Berzofsky, 1985; Rothbard & Taylor, 1988). The use of such peptides to stimulate T cells would be advantageous in the case of antigens like TPO which have been cloned and sequenced (Kimura *et al.*, 1987;

Correspondence: Dr S. M. McLachlan, Endocrine Immunology Unit, Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, Wales.

Libert *et al.*, 1987; Magnusson *et al.*, 1987) but are difficult to purify in large amounts in the absence of associated proteins such as thyroglobulin.

Antigen-specific stimulation of T cells involves multiple cellular interactions. Therefore, in order to maximize the possibility of detecting specific T cell responses to TPO and thyroglobulin *in vitro*, we have investigated proliferation using (i) lymphoid cells from different lymphoid organs; (ii) unfractionated lymphoid suspensions as well as fractions depleted of $CD8^+$ cells or enriched for autologous low-density cells (LDC) (predominantly dendritic cells); (iii) conventional 200-μl well cultures as well as 20-μl 'hanging-drop' microcultures; and (iv) intact TPO and thyroglobulin, denatured thyroglobulin and synthetic peptides predicted on the basis of the amino acid sequence of TPO to be T cell epitopes.

MATERIALS AND METHODS

Types of antigen

Intact thyroglobulin and TPO. Human TPO was extracted from deoxycholate solubilized thyroid microsomes using affinity chromatography based on monoclonal anti-porcine TPO antibodies (Ohtaki *et al.*, 1982; Czarnocka *et al.*, 1985). The final product had a specific activity of approximately 300 U/mg of protein, using the guiacol method of Hosoya & Morrison (1967) to determine enzyme activity and the method of Bradford (1967) to determine protein concentration. The preparations contained trace amounts of thyroglobulin (0·2% as determined by radioimmunoassay). Thyroglobulin was purified from Graves' disease thyroid tissue homogenates by ammonium sulphate precipitation (Derrien, Michel & Roche, 1948; Valenta *et al.*, 1968) followed by gel filtration on Sephacryl S-400.

Denatured thyroglobulin. Thyroglobulin was reduced and alkylated and subsequently digested as follows: 100 μl 0·1 M dithiothreitol in 2 M Tris–HCl, pH 8·2, were added to 25 mg freeze-dried thyroglobulin dissolved in 1 ml 8 M urea, pH 10. After incubation for 2 h at 20°C, 100 μl 0·5 M iodoacetamide in 2 M Tris–HCl, pH 8·2 were added. The mixture was dialysed against distilled water overnight and subsequently freeze-dried in aliquots. The reduced and alkylated thyroglobulin (13 mg) was dissolved in 1 ml 0·1 M $NaHCO_3$, pH 7·9, and treated with 264 μg bovine pancreas trypsin (Sigma Chemical Co., Poole, UK) for 3 h at 37°C, followed by 1·5 mg trypsin inhibitor (Sigma). After dialysis against distilled water overnight at 4°C, the digested thyroglobulin was freeze-dried in aliquots. For use in cultures, the reduced and alkylated thyroglobulin and digested thyroglobulin were resuspended in culture medium (described later) and sterilized by filtration (0·2 μm, Flow Laboratories, Irvine, UK). In addition to reduced and alkylated thyroglobulin and digested thyroglobulin, thyroglobulin was denatured by heating to 100°C for 30 min.

Synthetic TPO peptides. The algorithms of De Lisi & Berzofsky (1985) and Rothbard & Taylor (1988) were used to predict regions containing amphipathic helices and/or the characteristic structual motif likely to form potential T cell epitopes in the amino acid sequence of human TPO. In the absence of any prior information concerning areas of this large molecule likely to stimulate T cells, a random selection of 12 regions containing potential T cell epitopes was made. Peptides, 10–17 amino acids in length corresponding to these regions (Table 1), were synthesized manually by solid phase f-moc amino acid residue coupling (Kovacs *et al.*, 1985) using the RaMPS Multiple Peptide synthesis system (DuPont–NEN Products, Boston, MA). In addition, nine peptides lacking the features of potential epitopes were synthesized to provide controls. Amino acid composition of the synthetic peptides was determined following acid hydrolysis (University of California in San Francisco Bio-Molecular Resource Center).

Table 1. Sequences of 12 synthetic peptides (single letter amino acid code) predicted to be T cell epitopes on thyroid peroxidase (TPO) and nine control peptides

No.	Peptide sequence	Amino acid residues on TPO
1	LKKRGILSPAQLLS	62–75
2	SGVIARAAEIMETSIQ	84–99
B18	PPVREVTRHVIQVS	203–216
B8	PRQQMNGLTSFLDAS	313–327
3	LTALHTLWLREHNRL	403–417
4	HNRLAAALKALNAHW	414–428
B26	ARKVVGALHQIITL	437–450
B30	LPGLWLHQAFFSPWTL	514–529
5	MNEELTERLFVLSNSST	556–572
6	LDLASINLQRG	572–583
B5	RSVADKILDLYKHPDN	616–631
B6	IDVWLGGLAENFLP	632–645
Control peptides		
9	EARPAAGTAC	277–286
F1	CLPYMLPPKC	133–142
F2	CAPEPGNPGETRGP	375–388
B3/F5	EAFQQYVGPYEGYDSTA	461–477
10	YDSTANPTVSNV	473–484
11	RGGGLDPLIRGL	531–542
12	YNEWREFCGLPRLETPA	591–607
B7	GKFPEDFESCDSITGM	712–727
F3	CADPYELGDDGRTC	825–838

Rothbard epitopes are underlined and Berzovsky epitopes are indicated by a broken line above the relevant amino acids.

Types of Lymphoid Populations:

Unseparated suspensions from different lymphoid organs. Lymphocytes were extracted from peripheral blood of 17 patients with autoimmune thyroid disease (one with Graves' disease and 16 with Hashimoto's thyroiditis) and five healthy controls by density centrifugation (Böyum, 1976) on Lymphoprep (Nycomed, Birmingham, UK). Thyroid tissue and lymph nodes draining the thyroid were available at operation from five patients with autoimmune thyroid disease (two with Hashimoto's thyroiditis and three with Graves' disease) and thyroid tissue from one patient with a thyroid nodule. Lymphoid cells were extracted from lymph nodes by mechanical disaggregation and lysis of erythrocytes using buffered ammonium chloride or removal of erythrocytes and debris on Lymphoprep (Atherton *et al.*, 1985). Thyroid lymphocytes were isolated by digestion of the thyroid tissue using Dispase (Boehringer-Mannheim, Mannheim, FRG), incubation of the cell suspensions at 37°C overnight on tissue culture-treated Petri dishes to allow thyroid cells to adhere, followed by purification of the non-adherent cell

population on Lymphoprep (McLachlan *et al.*, 1986). In addition, as we have shown that lymphocyte populations extracted by mechanical disaggregation or digestion differ significantly in terms of cell surface markers and ability to secrete thyroid autoantibodies (McLachlan *et al.*, 1986), lymphocytes were isolated from thyroid tissue of two patients with Hashimoto's thyroiditis in three different ways: by digestion as outlined above; by mechanical disaggregation, followed by removal of dead cells and debris on Lymphoprep; or by digestion of the debris remaining after mechanical disaggregation. The viability of lymphoid cells, assessed by trypan blue dye exclusion (Boyse, Old & Chouroulinkov, 1964), was > 95% for blood lymphocytes and > 84% for lymph node and thyroid lymphocyte suspensions.

Suspensions depleted of CD8⁺ cells. Lymphocyte suspensions from blood, thyroid and lymph nodes were depleted of $CD8^+$ (suppressor/cytotoxic) cells by incubation with monoclonal antibody UHCT4 (Beverley, 1982) for 30 min at 4°C, followed by washing and exposure to Dynabeads and a magnet (Dynal, Wirral, UK). Control cells were treated in the same way after incubation in culture medium alone.

Isolation of T cells and low density cells. Peripheral blood T cells were obtained by rosetting with neuraminidase-treated sheep erythrocytes (Weiner, Bianco & Nussenzweig, 1973), separation of rosettes on Lymphoprep and lysis of erythrocytes using buffered NH_4Cl. LDC enriched for dendritic cells were isolated by adhering untreated lymphoid suspensions on to plastic Petri dishes overnight at 37°C, followed by centrifugation of the non-adherent cells on a hypertonic metrizamide (Nycomed) gradient as described by Knight *et al.* (1986).

Types of culture system

T cell responses, measured in terms of ^{3}H-thymidine (^{3}H-TdR) uptake by lymphoid cells incubated with or without different forms of thyroid antigen, were investigated using two types of culture system.

Assays in 200-μl wells. Two-hundred-microlitre aliquots of 1×10^5 lymphoid cells in culture medium (RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml fungizone and 2 mM L-glutamine, all from GIBCO, Paisley, UK, and 10% human A^+ plasma defibrinated using Arvin (Armour Pharmaceutical, Eastbourne, UK) were incubated at 37°C in the presence of 5% CO_2 with or without antigen (intact TPO, intact or denatured thyroglobulin, or synthetic TPO peptides). The following peptides were soluble in medium alone: 1, 10, 11, 12, B3/F5, B5, B6, B7, B8, 9, F2, F3 and F4. In contrast, peptides 2, 3, 4, 5, 6, B18, B26 and B30 were poorly soluble in culture medium and were therefore dissolved in dimethylformamide (DMF, Sigma) and responses to these peptides were assessed in comparison with control cultures containing 0·25% and 0·025% DMF (corresponding to the final DMF concentration in medium containing 25 and 2.5 μg/ml peptide). The mitogen concanavalin A (ConA, Sigma) was used as a positive control. Antigens or mitogens were tested in triplicate and six to nine replicates were set up to determine values for background incorporation. ^{3}H-TdR (specific activity 5 Ci/mM; 0·2 uCi/well, Amersham, UK) was added after 2–5 days and cultures were harvested 18 h later using an automated Skatron harvester and counted by liquid scintillation.

Assays in 20-μl hanging-drop microcultures. Lymphoid suspensions (unfractionated cells or 2×10^5–2×10^4 T cells alone or together with 500–2×10^3 LDC) were cultured in triplicate 20-μl hanging-drop microcultures with or without antigens (as described above) using RPMI 1640 (Dutch modification, Flow Laboratories) containing 10% A^+ serum, 50 μg/ml streptomycin and 2 mM L-glutamine. Terasaki plates (Nunc, Roskilde, Denmark) were inverted and cultured in humidified plastic boxes within a CO_2 (5%) incubator for 3–5 days. Cultures were pulsed with 1 μl/well ^{3}H-TdR (specific activity 2 Ci/mM, Amersham) for 2 h before harvesting by blotting onto filter discs and counting by liquid scintillation.

Expression of results

Proliferative responses were expressed as dpm ^{3}H-TdR incorporated or as a stimulation index, the ratio of ^{3}H-TdR uptake in the presence of antigen to ^{3}H-TdR uptake in culture medium alone.

Characterisation of thyroid autoantibody status of the donors of lymphoid tissue

TPO and thyroglobulin autoantibody levels were determined by ELISA (McLachlan *et al.*, 1982; Schardt *et al.*, 1982) in sera diluted 1/100, 1/1000 and 1/10 000. Sera that gave optical density readings < 0·100 in the ELISA at a dilution of 1/100 were considered to be negative. Sera giving readings > 0·250 at dilutions of 1/10 000 were considered to be highly positive.

Statistical analysis

Significance of differences between cultures with and without antigen was assessed by Student's *t*-test. When results were not normally distributed and sufficient replicate cultures were available, Wilcoxon's rank sum test was used.

RESULTS

Responses to thyroglobulin

Unfractionated lymphoid suspensions. Intact thyroglobulin: stimulation indices of > 2·0 were obtained for some blood lymphocyte suspensions from patients with Hashimoto's thyroiditis/Graves' disease incubated for 6 days with intact thyroglobulin in 200-μl cultures, for blood lymphocytes from one out of three control donors (donor no. 15, Table 2) and for lymph node lymphocytes from two thyroglobulin antibody-negative Graves' disease patients (donors 13 and 14) (Table 2). Although the triplicate values for ^{3}H-TdR incorporated were in some cases statistically different from those obtained for cultures in medium only, the significance of these results is not clear for the following reasons: (i) in several cases there was no relationship to the dose of thyroglobulin (stimulation index higher at 3 and 30 μg/ml than at the intermediate dose of 10 μg/ml); and (ii) individuals who were studied on more than one occasion, for example control donor no. 15, gave inconsistent results (data not shown). No proliferative responses were observed in cultures of Hashimoto's thyroiditis thyroid lymphocytes isolated by mechanical disaggregation and/or by digestion, despite the ability of B cells in suspensions from both patients to secrete relatively large amounts of thyroglobulin antibody over a 10 day culture interval (data not shown).

Denatured thyroglobulin: no enhancement in the proliferative response was observed using reduced and alkylated thyroglobulin compared with intact thyroglobulin, and

Table 2. Proliferative response to intact thyroglobulin (Tg), reduced and alkylated Tg (R&A Tg) and digested Tg by lymphocytes from peripheral blood (PBL) lymph node (LNL) and thyroid (TL) incubated for 6 days in 200-μl well cultures of lymphocytes suspensions from Tg antibody-positive ($TgAb^+$) or antibody-negative ($TgAb^-$) patients with Hashimoto's thyroiditis or Graves' disease, and control donors

Group/lymphocyte source	Donor no.	Stimulation index: Intact Tg (μg/ml) 3	Intact Tg (μg/ml) 10	Intact Tg (μg/ml) 30	R&A Tg (μg/ml) 3	R&A Tg (μg/ml) 30	Digested Tg (μg/ml) 3	Digested Tg (μg/ml) 30
$TgAb^+$ patients								
PBL	1	1·6*	—	2·1*	2·3	1·7	1·0	2·9
	2	2·3*	—	1·5	1·3	2·2*	1·0	2·3 (NS)
	3	1·0	—	0·8	0·9	1·3	1·4	0·7
	4	0·9	—	0·9	—	—	1·1	0·9
	5	2·3*	1·6	2·2*	—	—	—	—
	6	1·1	—	1·8	—	—	0·7	1·5
	7	2·2 (NS)	3·6 (NS)	2·0*	—	—	—	—
	8	1·3	1·1	1·4	—	—	—	—
LNL	9	0·8	0·9	0·7	—	—	—	—
TL	8a	0·8	1·1	0·9	—	—	—	—
	8b	0·9	1·0	0·8	—	—	—	—
	8c	1·0	0·9	0·8	—	—	—	—
	9a	1·0	0·9	0·7	—	—	—	—
	9b	0·9	0·7	0·5	—	—	—	—
	9c	1·2	1·0	0·7	—	—	—	—
$TgAb^-$ patients								
PBL	10	0·6	0·6	0·5	—	—	—	—
	11	0·8	1·0	1·0	—	—	—	—
	12	1·4	—	1·3	—	—	—	—
LNL	13	3·0*	1·6	1·3	—	—	—	—
	14	3·6*	1·4	3·2*	—	—	—	—
TL	14	0·5	0·4	0·1	—	—	—	—
Control donors	15	3·1†	—	2·1†	2·6†	1·9†	2·1†	2·6*
	16	0·9	1·0	1·0	—	—	—	—
	17	1·2	1·0	1·2	—	—	—	—

PBL from a patient 6 were depleted of CD8. TL were prepared by (a) mechanical disaggregation, (b) digestion, and (c) digestion of debris remaining after mechanical disaggregation. Significance of differences between triplicates with antigen, compared with medium only: *$P<0·05$ (Student's t-test); †$P<0·05$ (Wilcoxon rank sum test); NS, not significant; —, not determined.

although a slight increase in the response to digested thyroglobulin was observed in the case of one patient (donor 1), digested thyroglobulin did not stimulate lymphocytes from three other Hashimoto's thyroiditis/Graves' disease patients (Table 2). Virtually no intact thyroglobulin was present in reduced and alkylated thyroglobulin or digested thyroglobulin as deduced from the inability of thyroglobulin antibodies in Hashimoto's thyroiditis sera to bind to ELISA plates coated with these forms of thyroglobulin (data not shown).

T cells + autologous LDC. Intact thyroglobulin: a statistically significant ($P<0·001$) proliferative response to 10 μg/ml thyroglobulin was observed in 'hanging-drop' cultures of LDC + autologous T cells from a patient with Hashimoto's thyroiditis who had very high circulating levels of thyroglobulin antibody (Fig. 1a). Lower but still significant ($P<0·05$) levels of proliferation were also observed in the T cell fraction alone (Fig. 1a) but unfractionated PBL showed no response to thyroglobulin (data not shown). Similar responses to 0·5–50 μg/ml thyroglobulin were observed using cultures of T cells + LDC from two other Hashimoto's thyroiditis patients (illustrated for patient 21 in Fig. 1c). The response to thyroglobulin was detected on more than one occasion in patients 6 and 21. In contrast, cultures of T cells + LDC from four other Hashimoto's thyroiditis patients and two healthy control donors did not respond to thyroglobulin and a lack of response was confirmed for two of these patients on repeated investigation. T cells + LDC from some of these individuals proliferated at a low level in the presence of all concentrations of thyroglobulin (for example, patient 22, Fig. 1d) while in others the incorporation of ^{3}H-TdR was higher but extremely variable in the presence or absence of thyroglobulin as illustrated for a control donor in Fig. 1e.

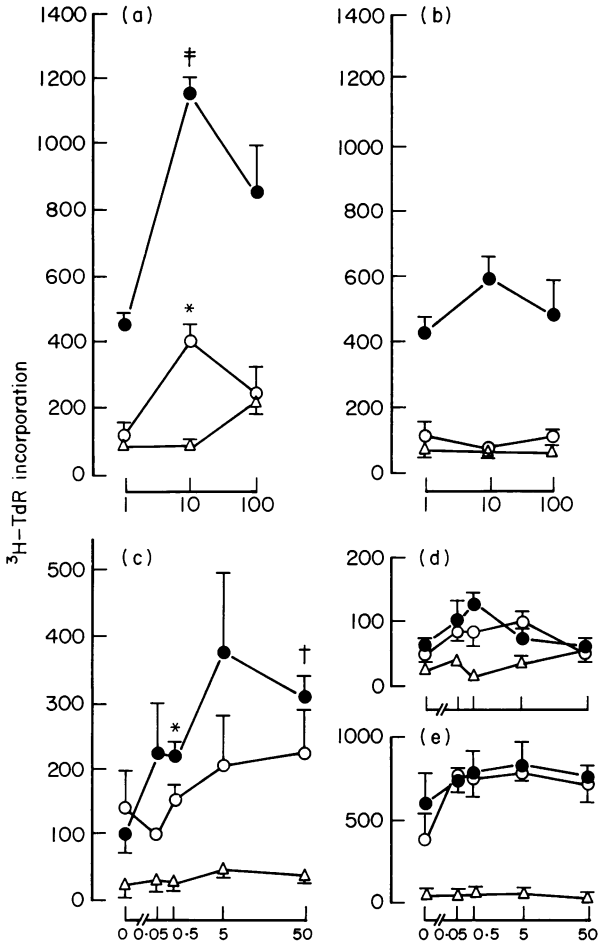

Fig. 1. Proliferative responses to intact thyroglobulin in 20-μl hanging-drop microcultures of peripheral blood T cells + autologous low-density cells (LDC) (●); T cells alone (○); or LDC alone (△) from patients with Hashimoto's thyroiditis (a, patient 6; c, patient 21; d, patient 22) and from a healthy control (e), and to heat-denatured thyroglobulin in patient 6 (b). Mean + s.e.m. ^{3}H-TdR incorporation (d/min) for triplicate cultures. The numbers of T cells and LDC per well were: a & b, 6×10^4 T cells and 1800 LDC; c & d, $1{\cdot}2 \times 10^5$ T cells and 500 LDC; e, $2{\cdot}1 \times 10^5$ T cells and 1000 LDC. Cultures were incubated for 4–5 days. Values significantly different from cultures incubated with 1 μg/ml thyroglobulin (a) or in medium only (c): $*P < 0{\cdot}05$; $\dagger P < 0{\cdot}01$; $\ddagger P < 0{\cdot}001$.

Denatured thyroglobulin: thyroglobulin denatured by boiling induced only low levels of proliferation by T cells + LDC from patient 6 (not significantly differently at 10 or 100 μg/ml compared with 1 μg/ml) and no response in the T cell fraction alone (Fig. 1b).

Proliferative responses to TPO

Intact TPO. Unfractionated lymphoid suspensions: only low levels of proliferation (stimulation indices $< 2{\cdot}0$) were observed in response to intact TPO in 200-μl well cultures of unfractionated lymphocyte suspensions (Table 3). In only one experiment, consisting of lymph node lymphocytes from a patient with Graves' disease, was a stimulation index $> 2{\cdot}0$ obtained, for which the incorporation of ^{3}H-TdR in triplicate cultures with and without TPO was significantly different. Statistically significant differences between replicates with and without antigen were observed for blood lymphocytes from

Table 3. Proliferative responses to intact thyroid peroxidase (TPO) by peripheral blood lymphocytes (PBL), lymph node lymphocytes (LNL) and thyroid lymphocytes (TL) from patients with Hashimoto's thyroiditis or Graves' disease, and controls, incubated in 200-μl well cultures for 6 days

Group/lymphocyte source	Donor no.	Stimulation index (TPO (μg/ml)) 2·5	25
Patients			
PBL	18	1·7*	1·5 (NS)
	1	0·9	0·8
	2	1·6†	1·9 (NS)
	23	1·6*	0·8
	4	0·5	0·5
LNL	19	2·4*	1·8 (NS)
	20	0·9	2·5 (NS)
	9	1·3	0·7
TL	20a	0·9	1·1
	9a	0·7	0·8
	9b	1·0	0·7
	9c	1·3	0·8
Control donor			
PBL	15	1·7*	1·4

Significance of differences between triplicate cultures with and without antigen: $*P < 0{\cdot}05$ (Student's t-test); $\dagger P < 0{\cdot}05$ (Wilcoxon rank sum test); NS, not significant.

Thyroid lymphocytes were isolated by (a) mechanical disaggregation; (b) digestion; (c) digestion of debris remaining after mechanical disaggregation.

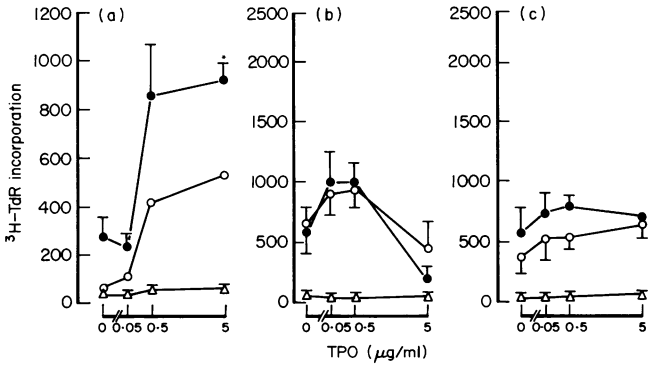

Fig. 2. Proliferative responses to intact thyroid peroxidase (TPO) in 20-μl hanging-drop microcultures of T cells + autologous low-density cells (LDC) (●); T cells alone (○); or LDC alone (△) from two patients with Hashimoto's thyroidits: a, patients 21, who had a high serum level of TPO antibody; b, patient 22, with a lower serum level of TPO antibody; and c, healthy control donor. Mean + s.e.m. ^{3}H-TdR incorporation (d/min) for triplicate cultures. Wells contained $2{\cdot}1 \times 10^5$ T cells and 1000 LDC and were incubated for 4–5 days. Values significantly different from those observed for cultures in medium only: $*P < 0{\cdot}01$.

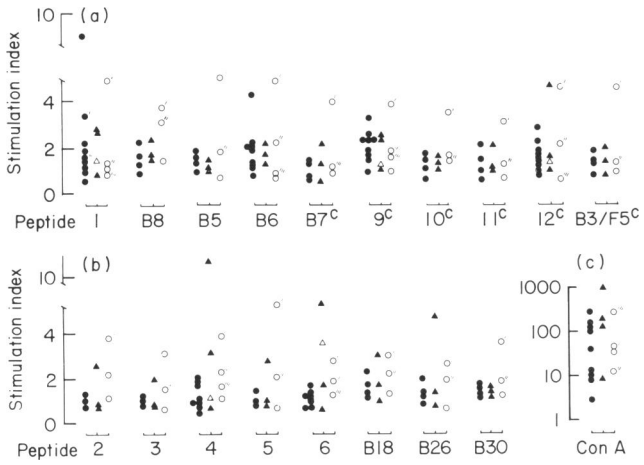

Fig. 3. Proliferative responses in 200-μl well cultures of lymphocytes from the blood, lymph nodes and thyroid tissue incubated for 6 days with: (a), four peptides containing potential T cell epitopes and six control peptides (c) soluble in culture medium; (b), eight peptides containing potential T cell epitopes soluble in DMF; and (c), concanavalin A (ConA). Results are expressed as a stimulation index, the ratio of ^{3}H-TdR incorporation with peptide to uptake in medium alone. Values are shown as the maximum response at 2·5 μg/ml or 25 μg/ml peptide and at 3·75 μg/ml Con A. Peripheral blood lymphocytes from patients with Hashimoto's thyroiditis or Graves' disease (●) or healthy donors (○); lymph node lymphocytes from patients with Hashimoto's thyroiditis or Graves' disease (▲) and thyroid lymphocytes from a patient without autoimmune thyroid disease undergoing surgery for a thyroid nodule (△). Replicate cultures obtained from a healthy donor on different occasions are indicated ○′, ○″ and ○‴.

three patients with Hashimoto's thyroiditis/Graves' disease, but the same level of stimulation (approximately 1·8) was observed for blood lymphocytes of a control donor (Table 3).

T cells+autologous LDC: in 20-μl hanging-drop microcultures, T cells from one patient with Hashimoto's thyroiditis with high serum levels of TPO antibody (detectable in serum diluted (1/10 000) proliferated significantly in response to 5 μg/ml TPO and proliferation was enhanced by autologous LDC (Fig. 2a). No proliferative response was detected using T cells+autologous LDC from another patient with Hashimoto's thyroiditis (Fig. 2b) with lower levels of serum TPO antibody (detectable at a dilution of 1/1000 but not at 1/10 000), nor did lymphocytes from a control donor respond to TPO (Fig. 2c).

TPO peptides. Unfractionated lymphoid suspensions: in the presence of the TPO peptides, lymphocytes from some patients as well as from one control donor on one occasion responded after incubation for 6 days in 200-μl well cultures, giving stimulation indices of 4·0 or above (Fig. 3). Significantly higher responses were observed in a few cases, by blood lymphocytes to peptide 1 and by lymph node lymphocytes to peptides 4 and 6. However, variable responses were obtained for blood lymphocytes isolated from one control donor on three separate occasions. Further, lymph node lymphocytes which gave a stimulation index of 11·4 when cultured immediately after isolation failed to respond to the same peptide after freezing and thawing.

Suspensions depleted of CD8$^+$ cells: in an attempt to enhance potentially significant responses to peptides, proliferation was assessed in lymphoid suspensions depleted of CD8$^+$ T cells by incubation of the suspensions with a monoclonal antibody (UCHT4) followed by exposure to magnetic beads. To control for effects of the depletion procedure, the response of this population was compared with the value obtained for suspensions exposed to 'Dynabeads' without prior incubation with monoclonal antibody. The response of thyroid lymphocyte suspensions from two patients with Graves' disease was unaffected by removal of CD8$^+$ cells. Modest enhancement of proliferation (two- to three-fold) was observed in CD8-depleted lymph node lymphocyte suspensions from two (out of two) patients in response to peptides 4, 6 and B6 (predicted to contain T cell epitopes). However, removal of CD8$^+$ cells also enhanced the response of some blood and lymph node lymphocytes to control peptides 9 and 12, to a comparable extent. Further, proliferative responses to all peptides investigated were enhanced two-fold in CD8-depleted lymphocyte suspensions from the blood of one control donor and thyroid lymphocytes from a patient without autoimmune thyroid disease undergoing surgery for a thyroid nodule.

DISCUSSION

In conventional microwell cultures, we have been unable to demonstrate proliferative responses to intact highly purified human thyroglobulin or TPO by lymphocyte suspensions from patients with Hashimoto's thyroiditis or Graves' disease which were significantly different from those observed in cultures of lymphocytes from some individuals (patients or control donors) without autoantibodies to thyroglobulin or TPO. Responses were not enhanced using lymphocytes from thyroid tissue and lymph nodes draining the thyroid gland, which we have shown contain a higher proportion of precursors able to secrete autoantibodies to thyroglobulin and/or TPO than the blood (Atherton *et al.*, 1985). The approach using different forms of antigen, including thyroglobulin denatured by reduction and alkylation or digestion, was also unsuccessful in stimulating lymphocyte proliferation in conventional 200-μl well cultures.

Our observations differ from those of other studies in which proliferative responses to intact thyroglobulin were detected in 200-μl well cultures of Hashimoto's thyroiditis but not normal T cells (Canonica *et al.*, 1984). The reasons for this discrepancy are not clear but may be related to unintentional differences in selection of patients or controls or to the use in our studies of highly purified thyroglobulin. The lack of response to digested thyroglobulin in our study is perhaps surprising, in view of studies of proliferation by murine T cell clones specific for human thyroglobulin which responded to small fragments of thyroglobulin produced by digestion (Male *et al.*, 1985). In addition, our results are in contrast with those of Shimojo *et al.* (1988) who showed that fragments of thyroglobulin prepared by digestion with V8 protease were stimulatory to lymphocytes from patients with chronic thyroiditis. However, as no data for healthy individuals were reported by Shimojo *et al.* (1988), the antigenic specificity of the response requires confirmation.

Twelve synthetic peptides predicted to be epitopes on TPO generally stimulated low-level responses in unfractionated lymphoid suspensions of some TPO antibody-positive or -negative patients or control donors. In another study (Fukuma *et al.*, 1990) we were unable to demonstrate proliferative responses to a peptide containing a potential T cell epitope shared by thyroglobulin and TPO (McLachlan & Rapoport, 1989).

Further, depletion of $CD8^+$ cells enhanced responses towards three TPO peptides predicted to be T cell epitopes and two control peptides, to a comparable extent in patients with or without TPO antibodies and in control donors without detectable levels of thyroid autoantibodies. Our observation that some synthetic TPO peptides stimulated low levels of proliferation in a proportion of individuals with and without serum TPO autoantibodies is comparable with the data of Harcourt *et al.* (1988) showing responses to several synthetic peptides, corresponding to regions of the alpha-subunit of the acetylcholine receptor, in cultures of lymphocytes from healthy individuals and acetylcholine receptor autoantibody-positive patients. As suggested by Harcourt & Jermy (1987), it is possible that the proliferative response by both myasthenic patients and healthy controls reflects a response to epitopes present on the acetylcholine receptor and on common exogenous antigens. A similar situation may apply to some synthetic TPO peptides and exogenous antigens. For example, the sequence of the potential T cell epitope on TPO shared with thyroglobulin was also homologous to sequences on two bacterial proteins (McLachlan & Rapoport, 1989). However, until it can be convincingly demonstrated that responses to such peptides are involved in the induction of acetylcholine-receptor autoantibody synthesis in myasthenia gravis, or TPO autoantibody synthesis in autoimmune thyroid disease, their relevance to the breakdown in tolerance to the acetylcholine receptor or to TPO remains questionable.

The TPO peptides used in this study were randomly selected from potential T cell epitopes covering a major portion of the large TPO molecule (100 kD). Very recently, a preliminary report has appeared (Dayan *et al.*, 1990) of the response of T cell lines and clones, generated by non-specific stimulation of *in vivo*-activated T cells extracted from Graves' disease thyroid tissue, to two TPO peptides. In this study, which was begun after our investigation was complete, the TPO peptides used to screen the T cell lines and clones included the panel described in Table 1 and the potential TPO–thyroglobulin epitope (all but one, B6, of which proved to be negative), as well as a second group of peptides, only one of which gave positive responses. If these responses are indeed specific for individuals with autoantibody reactivity to TPO, the combined results of our studies of primary cultures and those based on investigations of T cell lines or clones emphasize the difficulty of T cell epitope prediction on a large protein antigen.

In contrast to the lack of specific T cell responses we obtained using conventional 200-μl wells, we observed proliferation to intact thyroglobulin in hanging-drop microcultures of blood T cells + autologous LDC (enriched for dendritic cells) from several patients with Hashimoto's thyroiditis with high serum levels of autoantibodies to thyroglobulin. A response to TPO was also observed under the same conditions with T cells + LDC from one patient with Hashimoto's thyroiditis with a very high TPO autoantibody titre. No statistically significant proliferative responses to thyroglobulin/TPO were detected in hanging-drop cultures of T cells + autologous LDC from thyroglobulin/TPO antibody-negative individuals. Using a comparable system, Farrant *et al.* (1986) observed that T cells + fractions enriched for dendritic cells stimulated synthesis of thyroglobulin antibody by B cells from patients with Hashimoto's thyroiditis. As in our studies, the response was reproducible and limited to some but not all extremely high-titre thyroglobulin antibody-positive patients and was not detected in thyroglobulin antibody-negative individuals.

Dendritic cells appear to be the major accessory cells of the immune system (King & Katz, 1990) and their presence in chronically inflamed tissues, such as Graves' disease and Hashimoto's thyroiditis thyroid glands (Kabel *et al.*, 1988), suggests that they may be involved in the pathogenesis of autoimmune diseases. Further, dendritic cells can initiate experimental allergic encephalomyelitis and thyroiditis (Knight *et al.*, 1983, 1988). However, some T cell clones are unable to respond to antigen presented by dendritic cells (Katz *et al.*, 1986), possibly indicating limitations in their ability to process some antigens or deliver accessory signals for some T helper cells. In a few experiments we observed that suspensions enriched for Hashimoto T cells proliferated in respose to thyroglobulin in the absence of LDC. Previously, we demonstrated thyroglobulin antibody synthesis in some T cell enriched fractions, presumably because the density of some thyroglobulin antibody-secreting plasma cells is similar to that of T cell–sheep erythrocyte resettes (McLachlan *et al.*, 1983). The proliferative response was abrogated by heat denaturing thyroglobulin, a process which also destroyed the ability of the thyroglobulin to interact with thyroglobulin autoantibodies on an ELISA plate. Human autoantibodies to thyroglobulin appear to recognize conformational determinants on thyroglobulin (Male *et al.*, 1985). Consequently, it is possible that B cells with thyroglobulin antibody-surface receptors may be required for internalization and processing of thyroglobulin and possibly for thyroglobulin presentation to T cells, as described by Lanzavecchia (1985) for the proliferative response to tetanus toxoid. The nature of the cell which presents TPO/thyroglobulin to T cells in patients with autoantibodies to these thyroid antigens clearly requires further investigation.

The use of blood lymphoid cells fractionated into T cells and LDC has enabled us to detect proliferative responses to intact thyroglobulin or TPO which appear to be specific since they are only observed using lymphocytes from patients with autoantibodies to TPO/thyroglobulin. However, not all patients with Hashimoto's thyroiditis have T cells which proliferate in response to thyroglobulin or TPO in the presence of autologous LDC, and this suggests that the number of thyroid antigen-specific precursor T cells present in the peripheral blood is limiting. Consequently, the presence of sufficient precursor T cells, as well as the number and the type of antigen-presenting cells, are critical for investigations of human thyroglobulin-specific or TPO-specific proliferative responses.

ACKNOWLEDGMENTS

We are grateful to Dr Peter Beverley, ICRF Tumour Immunology Unit, University College and Middlesex School of Medicine, London, for his gift of UCHT4 antiserum, and to Dr Peter Byfield, Clinical Research Centre, London, for advice concerning digestion of thyroglobulin. Mrs Kathy Earlam provided excellent secretarial assistance. This work was supported by RSR, Cardiff and the Wellcome Trust (N.F., S.M.McL., D.I.Y. & B.R.S.), NIH grant DK 36182 (B.R.) and the Newcastle Area Health Authority (J.G. and S.M.).

REFERENCES

ALLEN, P.M. (1987) Antigen processing at the molecular level. *Immunol. Today*, **8**, 270.

ATHERTON, M.C., MCLACHLAN, S.M., PEGG, C.A.S., DICKINSON, A.,

BAYLIS, P., YOUNG, E.T., PROCTOR, S.J. & REES SMITH, B. (1985) Thyroid autoantibody synthesis by lymphocytes from different lymphoid organs: fractionation of B cells on density gradients. *Immunology*, **55**, 271.

BEVERLEY, P.C. (1982) The application of monoclonal antibodies to the typing and isolation of lympho-reticular cells. *Proc. R. Soc. Edinburgh Sect. B*, **81**, 211.

BJORKMAN, P.J., SAPER, M.A., SAMRAOUI, B., BENNETT, W.S., STROMINGER, J.L. & WILEY, W.C. (1987) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature*, **329**, 512.

BÖYUM, A. (1976) Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* **5**, (Suppl. 5), 9.

BOYSE, E.A., OLD, L.J. & CHOUROULINKOV, I. (1964) Cytotoxic test for demonstration of mouse antibody. *Methods med. Res.* **10**, 39.

BRADFORD, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72**, 248.

CANONICA, G.W., COSULICH, M.E., CROCI, R., FERRINI, S., BAGNASCO, M., DIRIENZO, W., FERRINI, O., BARGELLESI, A. & GIORDANO, G. (1984) Thyroglobulin-induced T cell *in vitro* proliferation in Hashimoto's thyroiditis: identification of the responsive subset and effects of monoclonal antibodies directed to Ia antigens. *Clin. Immunol. Immunopathol.* **32**, 132.

CZARNOCKA, B., RUF, J., FERRAND, M., CARAYON, P. & LISSITZKY, S. (1985) Purification of the human thyroid peroxidase and its identification as the thyroid microsomal antigen involved in autoimmune thyroid disease. *FEBS Lett.* **190**, 147.

DAYAN, C.M., LONDEI, M., CORCORAN, A.E., RAPOPORT, B. & FELDMANN, M. (1990) Specific recognition of thyroid peroxidase by thyroid infiltrating T cell clones in human autoimmune thyroiditis. *Colloq. INSERM*, **207**, 267.

DE LISI, C. & BERZOVSKY, J.A. (1985) T cell antigenic sites tend to be amphipathic structures. *Proc. natl Acad. sci. USA*, **82**, 7048.

DERRIEN, Y., MICHEL, R. & ROCHE, J. (1948) Recherches sur la preparation et les propriets de la thyroglobuline pure. *Biochim. biophys. Acta*, **2**, 245.

FARRANT, J., BRYANT, A.E., CHAN, J. & HIMSWORTH, R.L. (1986) Thyroglobulin-treated blood dendritic cells induce IgG anti-thyroglobulin antibody *in vitro* in Hashimoto's thyroiditis. *Clin. Immunol. Immunopathol.* **41**, 433.

FOROUHI, N.G., MCLACHLAN, S.M., MIDDLETON, S.L., ATHERTON, M.C., BAYLIS, P., CLARK, F. & REES SMITH, B. (1987) T cell regulation of thyroglobulin autoantibody IgG subclasses in Hashimoto's thyroiditis. *Clin. exp. Immunol.* **69**, 314.

FUKUMA, N., MCLACHLAN, S.M., PHILLIPS, D.I.Y., PEGG, C.A.S., RAPOPORT, B. & REES SMITH, B. (1990) T cell epitopes and thyroid peroxidase. In *Autoimmune Thyroiditis—Approaches Towards its Etiological Differentiation; Henning Symposium Schilddruse 1989* (ed. by W.A. Scherbaum, U. Bognor & G.F. Bottazzo) Springer-Verlag, Heidelberg (In press).

HARCOURT, G. & JERMY, A. (1987) Mapping the autoimmunizing epitopes on acetylcholine receptors. *Immunol. Today*, **8**, 319.

HARCOURT, G.C., SOMMER, N., ROTHBARD, J., WILLCOX, N.A. & NEWSOM-DAVIES, J. (1988) A juxta-membrane epitope on the human acetylcholine receptor recognised by T cells in myasthenia gravis. *J. clin. Invest.* **82**, 1295.

HOSOYA, T. & MORRISON, M. (1967) The isolation and purification of thyroid peroxidase. *J. biol. Chem.* **242**, 2828.

KABEL, P.J., VOORBIJ, H.A.M., DE HAAN, M., VAN DER GAAG, R.D. & DREXHAGE, H.A. (1988) Intrathyroidal dendritic cells. *J. clin. Endocrinol. Metab.* **65**, 199.

KATZ, D.R., FELDMANN, M., TEES, R. & SCHREIER, M.H. (1986) Heterogeneity of accessory cells interacting with T-helper clones. *Immunology*, **58**, 167.

KING, P.D. & KATZ, D.R. (1990) Mechanisms of dendritic cell function. *Immunol. Today*, **11**, 206.

KIMURA, S., KOTANI, T., MCBRIDE, O.M., UNICKI, K., HIRAI, K., NAKEYAMA, T. & & OHTAKI, S. (1987) Human thyroid peroxidase: complete cDNA and protein sequence, chromosome mapping and identification of two alternately spliced mRNA's. *Proc. natl Acad. Sci. USA*, **84**, 5555.

KNIGHT, S.C., MARTIN, J., STACKPOOLE, A. & CLARKE, J. (1983) Induction of immune responses *in vivo* with small numbers of veiled cells. *Proc. natl Acad. Sci. USA*, **80**, 6032.

KNIGHT, S.C., FARRANT, J., BRYANT, A., EDWARDS, A.J., BURMAN, S., LEVER, A., CLARKE, J. & WEBSTER, A.D.B. (1986) Non-adherent, low density cells from human peripheral blood contain dendritic cells and monocytes, both with veiled morphology. *Immunology*, **57**, 595.

KNIGHT, S.C., FARRANT, J., CHAN, J., BRYANT, A., BEDFORD, P.A. & BATEMAN, C. (1988) Induction of autoimmunity with dendritic cells: studies on thyroiditis in mice. *Clin. Immunol. Immunopathol.* **48**, 277.

KONG, Y., BAGNASCO, M. & CANONICA, G.W. (1986) How do T cells mediate autoimmune thyroiditis? *Immunol. Today*, **7**, 337.

KOVACS, J., KIM, S., HOLLERAN, E. & GORYCKI, P. (1985) Studies on the racemization and coupling of N^{α}, N^{im}-protected histidine active esters. *J. org. Chem.* **50**, 1497.

LANZAVECCHIA, A. (1985) Antigen specific interaction between T and B cells. *Nature*, **314**, 537.

LIBERT, F., RUF, J., LUDGATE, M., SWILLENS, S., ALEXANDER, N., VASSART, G. & DINSART, C. (1987) Complete nucleotide sequence of the human thyroid peroxidase–microsomal antigen cDNA. *Nucleic Acids Res.* **15**, 6735.

MACKENZIE, W.A., SCHWARTZ, A.E., FRIEDMAN, E.W. & DAVIES, T.F. (1987) Intrathyroidal T cell clones from patients with autoimmune thyroid disease. *J. clin. Endocrinol. Metab.* **64**, 818.

MAGNUSSON, R.P., CHAZENBALK, G.D., GESTAUTAS, J., SETO, P., FILETTI, S., DE GROOT, L. & RAPOPORT, B. (1987) Molecular cloning of the complementary deoxyribonucleic acid for human thyroid peroxidase. *Mol. Endocrinol.* **1**, 856.

MALE, D.K., CHAMPION, B.R., MATTHEWS, H. & SHEPHERD, P. (1985) Antigenic determinants of thyroglobulin differentiated using antigen fragments. *Immunology*, **54**, 419.

MCLACHLAN, S.M., CLARK, S., STIMSON, W.H., CLARK, F. & REES SMITH, B. (1982) Studies of thyroglobulin autoantibody synthesis using a micro-ELISA assay. *Immunol. Lett.* **4**, 27.

MCLACHLAN, S.M., DICKINSON, A., BAYLIS, P., PROCTOR, S. & REES SMITH, B. (1983) Enrichment and depletion of thyroglobulin antibody synthesising lymphocytes. *Clin. exp. Immunol.* **53**, 397.

MCLACHLAN, S.M., PEGG, C.A.S., ATHERTON, M.A., MIDDLETON, S.L., DICKINSON, A., CLARK, F., PROCTOR, S.J., PROUD, G. & REES SMITH, B. (1986) Subpopulations of thyroid autoantibody secreting lymphocytes in Graves' and Hashimoto thyroid glands. *Clin. exp. Immunol.* **65**, 319.

MCLACHLAN, S.M. & RAPOPORT, B. (1989) Evidence for a potential common T-cell epitope between human thyroid peroxidase and human thyroglobulin with implications for the pathogenesis of autoimmune thyroid disease. *Autoimmunity*, **5**, 101.

OHTAKI, S., MAKAGAWA, H., NAKAMURA, M. & YAMASAKI, I. (1982) Reactions of purified hog peroxidase with H_2O_2, tyrosine and methylmercaptoimidazole (goitrogen) in comparison with bovine lactoperoxidase. *J. biol. Chem.* **257**, 761.

ROTHBARD, J.B. & TAYLOR, N.R. (1988) A sequence pattern common to T cell epitopes. *EMBO J.* **1**, 93.

SCHARDT, C.W., MCLACHLAN, S.M., MATHESON, J. & REES SMITH, B. (1982) An enzyme-linked immunoassay for thyroid microsomal antibodies. *J. Immunol. Methods*, **55**, 155.

SHIMOJO, N., SAITO, K., KOHNO, Y., SASAKI, N., TARUTANI, O. & SAKAJIMA, H. (1988) Antigenic determinants on thyroglobulin: comparison of the reactivities of different thyroglobulin preparations

with serum antibodies and T cells of patients with chronic thyroiditis. *J. clin. Endocrinol. Metab.* **66,** 689.

STEINMAN, R.M., KAPLAN, G., WITMER, M.D. & COHEN, Z. (1979) Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers and maintenance in vitro. *J. exp. Med.* **149,** 1.

VALENTA, L., ROQUES, M., TORRESANI, J., ROLLAND, M. & LISSITSKY, S. (1968) Human thyroglobulin. Physico-chemical properties in relation to iodine content. *Biochim. biophys. Acta*, **168,** 507.

WEINER, M.E., BIANCO, C. & NUSSENZWEIG, V. (1973) Enhanced binding of neuraminidase-treated sheep erythrocytes to human T lymphocytes. *Blood*, **42,** 93.