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T cell responses to synthetic TSH receptor peptides in Graves' disease

N. TANDON, M. A. FREEMAN & A. P. WEETMAN *Department of Medicine, University of Sheffield, Clinical Sciences Centre, Northern General Hospital, Sheffield, UK*

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SUMMARY

Twenty-eight peptides, representing the entire extracellular domain of the TSH receptor, were synthesised to investigate which parts of this autoantigen may be targets for the T cell response in Graves' disease (GD). T cells from 11 of 21 controls and 26 of 36 newly diagnosed GD patients proliferated in response to one or more peptides with a stimulation index (SI) of $>2{\cdot}0$ ($\chi^2=2{\cdot}31$, $P>0{\cdot}1$). The response of patients and controls to any of the individual peptides was also not statistically different. However, individual patients gave high SIs with certain peptides to which controls either gave an absent or very weak response. HLA-DR3 was not associated with any particular response to TSHR peptides. Three out of seven GD patients whose T cells were evaluated before and after treatment showed a response of this kind only early in the course of their disease. Intrathyroidal T cells from four GD patients did not give a consistent proliferative response to pools of five peptides, and depleting peripheral blood T cells of their $CD8^+$ population did not affect the proliferative response. These results indicate that the T cell response to the TSH receptor in GD does not seem to be directed against any one particular epitope on the peptides we have tested which cover the extracellular domain.

Keywords Graves' disease T cell proliferation TSH receptor

INTRODUCTION

Patients with Graves' disease (GD) present with hyperthyroidism due to uncontrolled stimulation of thyroid hormonogenesis by antibodies which bind to the TSH receptor (TSHR) [1,2]. Investigation of the sites on the TSHR which bind these thyroid stimulating antibodies (TSAb) has been expedited by the molecular cloning of the human TSHR [3–5]. The extracellular region of the receptor comprises 418 amino acids and this is linked to a portion of 346 residues which contains seven transmembrane regions and shares considerable homology with the luteinizing hormone/chorionic gonadotrophin (LH/CG) receptor. Using site-directed mutagenesis, synthetic oligopeptides or chimaeric TSH-LH/CG receptors, several regions in the TSHR have been identified which are important to the interaction between the TSHR and TSH or TSAb [6–9] but in spite of these efforts, the TSAb binding site has not been fully delineated, and most likely this will depend on the tertiary structure of TSHR.

Much less is known about T cell epitopes on TSHR and indeed the T cell response to this key autoantigen in GD has hitherto been impossible to assess due to the lack of any suitable source of purified material. As $CD4^+$ T cell epitopes generally comprise linear peptides of around 12 amino acids, it is now feasible to probe the T cell response to TSHR in GD using a series of overlapping synthetic peptides. This report describes the T cell proliferative responses of a large group of GD patients to a set of 28 peptides corresponding to the entire extracellular region of the TSHR.

Correspondence: A. P. Weetman, Department of Medicine, University of Sheffield, Clinical Sciences Centre, Northern General Hospital, Sheffield S5 7AU, UK.

PATIENTS AND METHODS

Patients

Peripheral blood mononuclear cells (PBMC) were prepared from 36 newly diagnosed GD patients. The diagnosis of GD was made on the basis of biochemical hyperthyroidism, the presence of a diffuse goitre, in most cases with thyroid-associated ophthalmopathy, and the detection of thyroglobulin or microsomal antibodies. In the case of seven patients, PBMC from two different time points in the course of the disease (0 months and 6–12 months after carbimazole treatment) were also prepared. In an additional five GD patients, sufficient blood was obtained to prepare a CD8-depleted subset of T cells. Intrathyroidal lymphocytes were prepared from a further four patients who had been treated for at least 3 months before surgery with antithyroid drugs. Control PBMC were obtained from 21 healthy laboratory staff members with no family history of thyroid autoimmunity.

Preparation of PBMC

These were prepared by Ficoll–Hypaque density gradient centrifugation and washed three times before use.

Preparation of CD8-depleted T cells

T cells were separated from B cells by rosetting PBMC with a 2% suspension of aminoisoethyl thiouronium bromide (Sigma, Poole, UK)-treated sheep erythrocytes [10]. The non-rosetted cells (B cells and monocytes) were also collected, to be used subsequently as antigen-presenting cells in the proliferation assays. The T cells obtained by lysing the rosetted sheep erythrocytes with isotonic ammonium chloride were resuspended in RPMI-1640 (GIBCO, Paisley, UK) at a concentration of 10^7 cells/ml and incubated at 4°C for 30 min with OKT8 (mouse MoAb against the human CD8 molecule; Ortho, Raritan, NJ). After this incubation the cells were washed three times to remove free OKT8. Dynabeads (Dynal, Oslo, Norway), coated with sheep anti-mouse IgG, were washed 10 times using sterile PBS and incubated with the antibody- treated T cells at a bead to cell ratio of 10/1 for 30 min at 4°C. After this incubation, the $CD8^+$ population was removed by two 4-min periods of contact with a magnetic particle concentrator (Dynal). The remaining cells were washed three times before use.

Preparation of intrathyroidal lymphocytes

Primary cultures from thyroid follicles were prepared from the GD thyroidectomy specimens as detailed elsewhere [11]. Briefly, the specimens were minced with scissors and digested with a mixture of collagenase (1 mg/ml; Sigma) and dispase (4·5 mg/ml; Boehringer Mannheim, Lewes, UK). Semi-digested follicles were removed at 20-min periods for 2 h and then allowed to adhere to 75 cm^2 flasks by culture overnight at 37°C and 5% CO_2:95% air in RPMI-1640 medium with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). After overnight culture, the medium containing non-adherent cells was removed and subjected to Ficoll–Hypaque gradient centrifugation to provide intrathyroidal lymphocytes with less than 5% contaminating thyroid follicular cells.

Peptides

Twenty-eight peptides, each 20 amino acids in length, and overlapped by six residues (sequences shown in Table 1) spanning the entire extracellular domain of the TSHR were analysed, as well as a peptide corresponding to a sequence from mouse liver F protein (sequence: EFGYWDKGPKPERGR FLHRH) [12], kindly donated by Dr D. Oliveira, which was used as a control. All were made manually by the method of simultaneous multiple peptide synthesis [13,14]. The purity was always greater than 60% and usually greater than 85% by HPLC.

Culture conditions

PBMC and intrathyroidal lymphocytes were cultured in triplicate at a concentration of 10^5/well (final volume 200 μl) in 96-well, flat-bottomed plates (Costar, Cambridge, MA). Culture medium was as for the thyroid cultures detailed above. Peptides were dissolved at a concentration of 1 mg/ml in dimethyl sulphoxide (DMSO) and diluted down in medium to a concentration of 50 μg/ml. They were used at a final concentration of 5 μg/ml. Preliminary experiments showed that DMSO present at up to 2% in the culture medium did not affect the viability and proliferation of T cells to mitogens. Phytohaemagglutinin (PHA; Sigma) was used as a positive control to ensure that individual PBMC were capable of proliferation. The cells were cultured for 5 days at 37°C in 5% CO_2 in air. Sixteen hours before termination, ^{3}H-thymidine (Amersham International, Amersham, UK) was added, 1 μCi/well, and the incorporation was measured by harvesting onto glass fibre filters and liquid scintillation counting. Results were expressed as a stimulation index (SI) as follows:

$$SI = \frac{\text{mean ct/min with TSHR peptide}}{\text{mean ct/min with control peptide}}$$

Owing to paucity of cell numbers, CD8-depleted T cells were tested against pools of peptides at a final concentration of 5 μg/ml (six pools, five of five peptides each and one of three peptides). For these experiments, autologous B cells and monocytes were irradiated (30 Gy) and used as antigen-presenting cells (2×10^4 cells for 10^5 CD8-depleted T cells). The remainder of the proliferation assay was carried out in the same way as for the PBMC. The yield of intrathyroidal lymphocytes was also low and therefore these were tested for proliferation against the same peptide pools as the CD8-depleted T cells.

Table 1. Amino acid sequence of TSH receptor (TSHR) peptides

Peptide no.	Amino acid sequence	Residues of the TSHR
1	GGMGCSSPPCECHQEEDFRV	20–39*
2	EEDFRVTCKDIQRIPSLPPS	34–53
3	PSLPPSTQTLKLIETHLRTI	48–67
4	THLRTIPSHAFSNLPNISRI	62–81
5	PNISRIYVSIDLTLQQLESH	76–95
6	QQLESHSFYNLSKVTHIEIR	90–109
7	THIEIRNTRNLTYIDPDALK	104–123
8	DPDALKELPLLKFLGIFNTG	118–137
9	GIFNTGLKMFPDLTKVYSTD	132–151
10	KVYSTDIFFILEITDNPYMT	146–165
11	DNPYMTSIPVNAFQGLCNET	160–179
12	GLCNETLTLKLYNNGFTSVQ	174–193
13	GFTSVQGYAFNGTKLDAVYL	188–207
14	LDAVYLNKNKYLTVIDKDAF	202–221
15	IDKDAFGGVYSGPSLLDVSQ	216–235
16	LLDVSQTSVTALPSKGLEHL	230–249
17	KGLEHLKELIARNTWTLKKL	244–263
18	WTLKKLPLSLSFLHLTRADL	258–277
19	LTRADLSYPSHCCAFKNQKK	272–291
20	FKNQKKIRGILESLMCNESS	286–305
21	MCNESSMQSLRQRKSVNALN	300–319
22	SVNALNSPLHQEYEENLGDS	314–333
23	ENLGDSIVGYKEKSKFQDTH	328–347
24	KFQDTHNNAHYYVFFEEQED	342–361
25	FEEQEDEIIGFGQELKNPQE	356–375
26	LKNPQEETLQAFDSHYDYTI	370–389
27	HYDYTICGDSEDMVCTPKSD	384–403
28	CTPKSDEFNPCEDIMGYKFL	398–417

* Residues designated 1–19 are the signal sequence; residue 20 is the first amino acid of the functional receptor.

HLA-typing

For HLA-DR typing, genomic DNA was prepared from patients and DR types assigned by restriction fragment polymorphism analysis as described elsewhere [15,16]. Briefly, DNA was digested with *Taq* I, electrophoresed and Southern blotted onto nylon membranes. These filters were then hybridized sequentially with DQA and DRB probes labelled with ^{32}P by random hexanucleotide priming. After stringent washing, filters were exposed for 2–10 days with Kodak XAR-5 film. DR types were then identified by visual inspection.

RESULTS

PBMC responses

The response of PBMC from normal subjects ($n=21$) and GD patients ($n=36$) is shown in Fig. 1; for clarity only those SI values greater than 2·0 are shown. All PBMC preparations proliferated in response to PHA. Fourteen (39%) of the GD patients failed to give a response of this magnitude to any of the peptides, compared with 10 (48%) of the controls; the remaining subjects responded to one or more peptides, up to a maximum of nine in two GD patients. Taking a SI of greater than 2·0 to any peptide as a positive response, there was no significant difference between the GD and control cultures ($\chi^2=2{\cdot}31$, $P>0{\cdot}1$). If a SI of greater than 3·0 is considered as a positive response, nine of the controls (43%) and 18 of the GD patients (50%) reacted to one or more peptides, again not a significant difference ($\chi^2=0{\cdot}27$, $P>0{\cdot}1$).

When the responses to individual peptides were evaluated, there were no significant differences between the SI produced by controls and GD patients for any of the 28 peptides ($P>0{\cdot}05$ by Wilcoxon test, all comparisons). The percentage of subjects responding to each peptide is shown in Table 2; there was no significant difference in the frequency of positive responses (SI > 2·0) for any of the peptides ($P>0{\cdot}05$ by χ^2 test, all comparisons). Despite this overlap between the two groups of subjects, there were two peptides (11 and 12) which produced a response in over 30% of the GD patients and 16 (44%) patients responded to one or other peptide with a SI of greater than 2·0; SI values greater than 8·0 were seen in four patients. Other individual patients also gave high SI values with certain peptides, to which control subjects either failed to respond or gave relatively low responses, particularly peptides 4, 10, 14, 16, 17, 19 and 25.

Seven patients were studied before and after a course of antithyroid drugs (Table 3): it was obviously not possible to withhold treatment from patients for repeated studies of the responses in order to establish their consistency. In one patient there was no response before or after treatment, in four the proliferative response disappeared or diminished, while in the remaining two there was partial overlap of the peptides which produced a T cell response, with greater responses after antithyroid drugs.

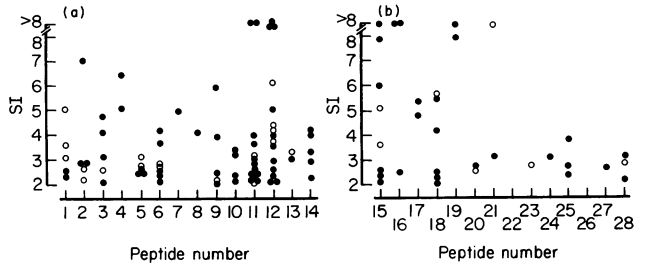

Fig. 1. T cell proliferative response to TSH receptor (TSHR) peptides 14 (a) and 15–28 (b). For clarity only those subjects giving a stimulation index (SI) of greater than 2·0 are shown. ●, Patients with Graves' disease ($n=36$ tested against each peptide); ○, normal controls ($n=21$ tested against each peptide).

Correlation of responses with HLA-DR type

DNA for HLA-DR typing was obtained from the last 23 of the patients studied. Thirteen (57%) of these were DR3$^+$, in keeping with the known association of this allele with GD (33% of 100 local control subjects were HLA-DR3$^+$; $\chi^2=4{\cdot}4$, $P<0{\cdot}05$ by 2×2 contingency tables). Five (39%) of the DR3$^+$ subjects and four (40%) of those without DR3 did not produce a response (SI > 2·0) to any peptide. There was no obvious difference between these two groups of patients in their response to any single peptide, or between other DR types and responses.

CD8-depleted T cell responses

The CD8-depleted population obtained after fractionation with magnetic beads always contained fewer than 4% CD8$^+$ cells and usually less than 2%. The results with these cells are shown in

Table 2. Percentage of control subjects and Graves' disease (GD) patients with peripheral blood mononuclear cells (PBMC) giving a proliferative response (stimulation index greater than 2·0) with various TSH receptor (TSHR) peptides

Peptide no.	Percentage giving SI > 2·0	
	Controls ($n=21$)	Graves' disease ($n=36$)
1	14	6
2	10	8
3	5	11
4	0	6
5	10	8
6	10	14
7	0	3
8	0	3
9	5	11
10	0	11
11	10	33
12	19	31
13	5	3
14	0	14
15	10	16
16	0	8
17	0	6
18	5	14
19	0	6
20	5	3
21	5	3
22	0	0
23	5	0
24	0	3
25	0	8
26	0	0
27	0	3
28	5	6

Table 3. T cell proliferative responses to TSH receptor (TSHR) peptides before and after anti-thyroid drug treatment in seven patients with Graves' disease

Patient	Peptides producing T cell proliferation (SI)*	
	Before treatment	After treatment
1	Nil	Nil
2	14 (4·0)	Nil
3	11 (2·5)	11 (2·1)
4	2 (2·9); 6 (2·3); 9 (3·9)	6 (3·6); 7 (4·9)
5	10 (2·3); 11 (2·7); 12 (2·2); 14 (2·2)	Nil
6	5 (2·4); 11 (2·4); 18 (2·2); 19 (8·0)	5 (2·3); 18 (5·6); 24 (3·0)
7	11 (3·6); 12 (8·5); 17 (5·3); 18 (4·3); 31 (5·8)	Nil

* The number of each peptide producing a stimulation index > 2·0 is given, followed by the SI in parentheses.

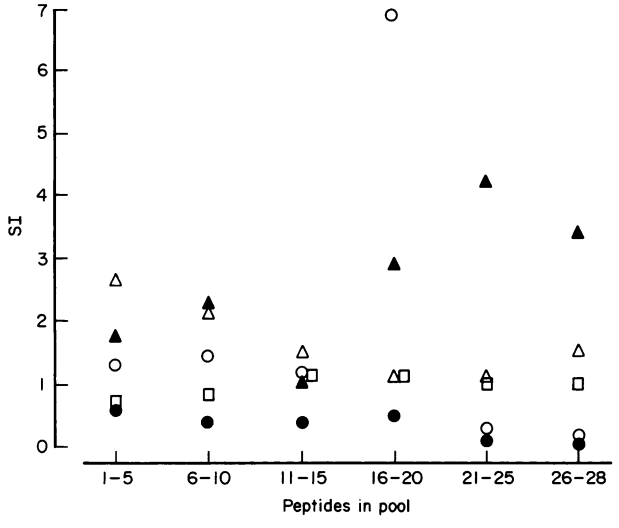

Fig. 2. Proliferative responses of CD8-depleted T cells from five patients with Graves' disease to six pools of TSH receptor (TSHR) peptides. The numbers of the peptides in each pool is given on the abscissa.

Table 4. Response of intrathyroidal lymphocytes from four Graves' disease (GD) patients to pools of TSH receptor (TSHR) peptides

Patient	SI with peptide pool*					
	1–5	6–10	11–15	16–20	21–25	26–28
1	1·7	0·7	1·0	0·7	0·7	1·0
2	3·0	1·1	1·5	1·1	0·6	0·6
3	1·7	0·9	1·2	0·7	1·6	1·0
4	0·8	3·5	2·3	1·1	0·9	0·9

* Proliferation is shown as the stimulation index (SI) in response to each pool of peptides.

Fig. 2. Insufficient sample remained to compare these responses with those by PBMC from the same individual and pools of three or five peptides were used to stimuate the T cells. Despite these caveats, it seems that CD8-depletion had little effect on the magnitude or frequency of T cell responses to TSHR peptides, with only two individuals producing a SI greater than 3·0. In one of these, the SI was 6·9 with the pool of peptides numbers 16–20 but was lower than 1·5 with all other pools, suggesting that this particular pool contained one or more critical peptides. However, similar instances of a single high response were observed with unfractionated PBMC, suggesting that $CD8^+$ cells were not responsible for limiting the reactivity of GD T cells in those experiments.

Intrathyroidal lymphocyte responses

These are shown in Table 4. Weak responses were observed in two of the four patients, in patient 2 in response to peptides 1–5, and in patient 4 in response to peptides 6–10 and 11–15. The other two patients failed to give a SI greater than 2·0.

DISCUSSION

Since the cloning of the human TSHR [3–5], there has been considerable interest in the delineation of B cell epitopes on this key autoantigen in GD. However, B cell epitopes are generally complex as they depend heavily on the tertiary structure of the antigen: linear peptides derived from antigen sequences have limited value in determining the region of antibody binding, as the sites identified by the use of such material are likely to represent cryptotopes or unfoldons [17] and sequential TSHR epitopes do not appear to be recognized by TSAb [18]. However, short, synthetic peptides have been used in binding assays and in experiments to inhibit TSAb binding [6–8]. These results, combined with those from deletion/substitution changes in the TSHR [9], have suggested that sequences 32–56, 123–131, 309–317 and 333–343 may be important for TSAb binding, but such findings remain preliminary. Studies with chimaeric TSH-LH/CG receptors indicate still greater complexity of TSAb binding, which utilizes both distal and proximal domains of the extracellular portion of the TSHR and differs from the binding sites used by TSH [19].

In contrast, T cell epitopes which bind to MHC class II molecules and are recognized by $CD4^+$ T cells are usually linear peptide sequences of around 12 amino acids in length. Although algorithms have been devised to predict motifs within a protein sequence which are potential T cell epitopes [20,21], their use has not been universally successful in identifying all of the important epitopes in a molecule. Indeed, we recently used one such algorithm to delineate T cell epitopes on another thyroid autoantigen, thyroid peroxidase (TPO), but found no close correlation between the occurrence of predicted motifs and the ability of synthetic peptides to stimulate T cells from patients with autoimmune thyroiditis [22]. For this reason, we chose in the present study to construct a series of overlapping peptides covering the entire extracellular domain of the TSHR (residues 20–417) in an attempt to define T cell epitopes on this molecule. To limit the number of peptides for assay we chose an overlap of six amino acids although to cover all possible epitopes the peptides should overlap by 11. It is, therefore, possible that important TSHR epitopes exist which have not been tested in the present study. It is also possible that T cells in GD patients

may also recognize epitopes in the region which is part of the TSHR distal to residue 417, but this contains seven transmembrane sequences and an intracytoplasmic portion with close sequence homology to other G protein-coupled receptors and therefore seems less likely to be of importance in generating a T cell response in GD.

Bearing in mind these limitations, our results fail to show a consistent T cell response to the potential epitopes tested, despite selecting a large number of newly diagnosed GD patients. Control subjects also responded to some of the TSHR peptides, a phenomenon which has been noted with peptides derived from other autoantigens such as the acetylcholine receptor and TPO [22,23]. Whether these responses by otherwise healthy subjects constitute fortuitous cross-reactivity or represent a more fundamental and generalized recognition of self-antigen is unclear. Despite the lack of any statistically significant difference in responses between patients and controls, individual responses by GD patients to certain TSHR peptides (e.g. 4, 14, 16, 17 and 19) were of such a magnitude, compared with the response of control subjects and the remaining patients, that it seems likely these represented epitopes recognized by T cells in a minority of patients. Similar, heterogeneous responses were observed with a panel of TPO peptides in autoimmune thyroiditis [22] and even within an individual, T cell clones have been shown to recognize diverse TPO epitopes [24]. There were no clear differences between HLA-DR3-positive and -negative patients in their responses but our sample size was very small.

Two possible explanations for the weak and inconsistent PBMC responses are that this population has a low frequency of TSHR-specific T cells and that any such cells are under active suppression, as cogently argued by Volpé [25]. We attempted to test the first of these possibilities by using intrathyroidal lymphocytes from four GD patients as these contain a high proportion of autoantigen-specific T cells [26,27]. Sufficient lymphocytes were obtained to assess the response to pools of peptides. No enhanced proliferation was seen (compared with the PBMC experiments), but this may have been due to prior anti-thyroid drug treatment; these agents have a variety of immunological effects, including amelioration of thyroiditis [28,29] and in four of six responding patients tested, the PBMC response to TSHR declined or disappeared (Table 3). In one recent study, T cell responses to a 38-kD β-cell autoantigen in type 1 diabetes could only be elicited after an initial cycle of expansion in the presence of IL-2 [30]. It will be worth using this strategy in future experiments to overcome problems of low precursor frequency, although this has not proved necessary in detecting peripheral blood T cell responses to glutamate decarboxylase in diabetes [31]. Direct measurement of precursor frequency using peptides would be an alternative approach, but this cannot easily be used for screening the large number of peptides in this study. To address the second possibility of active suppression, we removed $CD8^+$ cells from purified T cells, a procedure we have previously shown to enhance the response of $CD4^+$ T cells to thyroglobulin and TPO in patients with Hashimoto's thyroiditis [32]. No single peptide pool gave a consistent response with the five patients tested.

We have been unable to find a single peptide in this series which produces T cell stimulation in all GD patients but not controls. This may be due to a low frequency of responding T cells in the PBMC and intrathyroidal populations, the latter studied after anti-thyroid drug therapy, or it could be the result of failing to test all possible TSHR epitopes, so that those which are important have been missed. Several peptides gave reasonable SI values with a minority of GD patients, similar to our previous findings with a panel of TPO peptides [22], suggesting that the T cell response to TSHR in GD is heterogeneous.

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