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T cell responses to orbital antigens in thyroid-associated ophthalmopathy

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SUMMARY

Thyroid-associated ophthalmopathy (TAO) is most likely to be a T cell-mediated disease, in which cytokines released in the extraocular muscles activate fibroblasts, increasing glycosaminoglycan production. The nature of the orbital antigen recognized by the infiltrating T cells is unclear, although it is possible that there is cross-reactivity between this and a thyroid autoantigen to explain the close association with thyroid autoimmunity. We have tested the ability of human and porcine eye muscle antigen preparations to stimulate proliferation of circulating T cells from healthy subjects and patients with TAO or Graves' disease without clinical TAO. Occasional responses were seen, particularly after depletion of $CD8^+$ T cells, and two out of 10 TAO patients responded to eye muscle proteins of 25–50 kD after fractionation of antigens on gels and subsequent elution. There was no disease-specific response of T cells to R1, R14, D1 and 1D3, recombinant proteins identified from screening an eye muscle cDNA library with sera from patients with autoimmune thyroid disease. We have also found that interferon-gamma (IFN-γ) production by T cells from TAO patients was not stimulated by eye muscle membrane antigens or by 1D3. These results suggest that the frequency of circulating T cells responding to eye muscle antigens in TAO is low, and that several candidate orbital antigens, including the 64-kD protein 1D3, are unlikely to be important T cell autoantigens in this condition.

Keywords Graves' disease thyroid-associated ophthalmopathy T cell responses

INTRODUCTION

Thyroid-associated ophthalmopathy (TAO) is a frequent clinical and subclinical finding in patients with Graves' disease: it may also occur in patients with Hashimoto's thyroiditis and even in ostensibly euthyroid patients, although many of these turn out to have subtle evidence of underlying autoimmune thyroiditis [1]. The nature of this association is unclear, but one possibility is the sharing of a cross-reactive autoantigen in the orbit and the thyroid. The underlying pathology in TAO is infiltration of the extraocular muscles by lymphocytes, particularly activated T cells, with fibroblast activation, leading to glycosaminoglycan (GAG) accumulation, oedema and finally fibrosis [2–5]. Cytokines, known to be released by infiltrating mononuclear cells [6], can stimulate fibroblasts to secrete GAG *in vitro*, and orbital fibroblasts are more sensitive to these cytokines than fibroblasts from other sites, suggesting a reason for the distinctive anatomical localization of TAO [7,8]. T cell-derived cytokines can induce expression of intercellular adhesion molecule-1 (ICAM-1), HLA-DR and heat shock protein by extraocular muscle fibroblasts, and these surface molecules may also have a role in pathogenesis [9–11].

It is likely, therefore, that TAO is at least in part a T cell-mediated disease. Although a considerable effort has been made to identify eye muscle-specific autoantibodies, there is no current consensus that these exist: most of the available evidence suggests that the antibodies detected to date are non-specific and arise as a result of eye muscle injury [12]. Comparatively few studies have assessed the T cell response to orbital antigens in TAO, yet identification of the autoantigen(s) recognized by T cells would further our understanding of the pathogenesis of TAO, particularly regarding the existence of a cross-reactive antigen in the thyroid and the orbit. We have therefore analysed T cell responses in TAO to fractionated eye muscle membrane antigens (eluted from polyacrylamide gels) and to potential recombinant antigens (solubilized from nitrocellulose blots or as fusion proteins) derived from screening an orbital cDNA library and detailed in Table 1 [13].

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MATERIALS AND METHODS

Subjects

Patients with Graves' disease and no clinical evidence of TAO

Table 1. Summary of recombinant proteins used as stimulatory and control antigens

Recombinant	Isolated from (cDNA library)	Size/homology	Employed as
R1	Eye muscle	1·0 kb/347aa Fragment unknown	Lysogen and on nitrocellulose
R14	Eye muscle	0·7 kb/193aa Carboxyl half of IgE binding protein	Nitrocellulose
D1	Thyroid	0·3 kb/98aa Fragment unknown	Lysogen
1D3	Thyroid	64 kD/573aa Entire coding sequence	Membrane of eukaryotic cells expressing 1D3
I3 (control)	Placenta	0·3 kb/100aa Fragment unknown	Nitrocellulose
Neo (control)	–	–	Membrane of eukaryotic cells expressing neomycin resistance gene

and patients with TAO of grade III severity or greater [14] were selected from a single endocrine clinic. All TAO patients were within 3 years of the onset of symptoms: none was taking immunosuppressive agents, although two had received steroids previously. Normal subjects were laboratory staff with no personal history of thyroid disease.

Eluted human eye muscle antigen preparation

Human eye muscle tissue was homogenized in 10 mM Tris/50 mM NaCl pH 7·4 with protease inhibitors (PMSF, TLCK, TPCK, pepstatin A; all from Sigma, Poole, UK) and spun at 800 *g* for 10 min. The supernatant was harvested and spun at 10 000 *g* for 20 min and again at 100 000 *g* for 60 min. The final supernatant was aliquoted and stored at −80°C until use. One aliquot (2·4 mg/ml) was run down a 16 × 16 cm, 12% SDS polyacrylamide gel together with prestained low molecular weight markers, for 4 h at 150 V. A portion of the gel and the marker lane were removed, stained with coomassie blue and dried as a reference to estimate the apparent molecular weight of the fractions. The remainder of the gel was measured and placed to equilibrate in 20 mM Tris pH 8·0 for 30 min. A gel elution apparatus (Techne Ltd., Duxford, UK) was set up as described by Persidis & Harcome [15] and the gel was eluted for 3 h at 5 V, the current being reversed for 10 s before switching off to release protein stuck to the dialysis membrane. Fractions were collected using a multichannel pipette, 200 μl being removed from each well. The fractions from each row beneath the gel were pooled and stored at −80°C until use. A Bradford protein assay was used to determine the protein concentration in each fraction, and the fractions were adjusted to equal concentration. The samples were UV irradiated for 30 min and 20 μl of each fraction were added, in triplicate, to the proliferation assay. Tris elution buffer taken from wells not covered by the gel was used as a control.

Preparation of solubilized recombinant proteins from nitrocellulose filters

Antigen was prepared from nitrocellulose filters saturated with the recombinant proteins R1 and R14 (from an eye muscle cDNA library) and I3 (a control antigen from a placental cDNA library) using the method described by Abou-Zeid *et al.* [16]. Each cm^2 piece of filter was solubilized in 600 μl of dimethyl sulphoxide for 2 h at room temperature and then resuspended in particulate form by adding 500 μl of carbonate buffer pH 9·6. The particulate antigen was washed in buffer and resuspended in RPMI 1640. The test and control proteins were added to peripheral blood mononuclear cells (PBMC) in proliferation assays in replicates of six, at two dilutions: 20 μl of a 1:100 dilution or of the undiluted suspension per 200 μl.

Purified recombinant proteins

Recombinant eye muscle proteins (D1 and R1) were prepared by expanding lysogen in LB medium, subjecting the cultures to thermic shock (20 min, 45°C) and adding isopropylthio-βD-galactoside (IPTG) (Nova Biochem, Nottingham, UK) to a final concentration of 10 mM. The cells were pelleted by centrifugation at 1000 *g* for 10 min at 4°C and then resuspended in 5 ml of buffer containing 100 mM Tris, 10 mM EDTA and 1 mM PMSF pH 7·5. Between 1 ml and 2 ml of this crude lysate was sonicated, sheared by passing repeatedly through a 21 G needle, and finally Nonidet p40 (BDH, Lutterworth, UK) was added to a final concentration of 1% v/v. After rinsing well using an end-over-end rotation for 15–20 min, the volume was made up to 4 ml with 10 mM Tris, 1 mM NaCl pH 7·5. The treated lysate was applied on a 1-ml Protosorb 'lac Z' immunoaffinity adsorbent column (Promega, Southampton, UK) and the recombinant protein eluted using three washes of 1 ml of 0·1 M carbonate/bicarbonate buffer pH 10·8. The eluates from the three washes were pooled, and the presence of the recombinant protein confirmed by running an aliquot on a 7·5% acrylamide gel (giving an isolated band of 115 kD). The

remaining protein was frozen at −80°C until use. Before adding the proteins to the PBMC proliferation assay, they were dialysed against culture medium over a 24-h period at 4°C and then sterilized using a 0·22 μm filter. The proteins were added to the PBMC in triplicate at final concentrations of 2 and 4 μg/well. The controls were wells without added antigen.

Eye muscle and Chinese hamster ovary (CHO) membrane antigens

Human eye muscle (HEM), pig eye and pig skeletal muscle and CHO cell membrane preparations were also used as test and control antigens. These were prepared by homogenizing the tissue in 10 mM Tris/50 mM NaCl pH 7·4 with protease inhibitors (PMSF, TLCK, TPCK, pepstatin A) and spinning at 800 *g* for 10 min. The supernatant was harvested and spun at 10 000 *g* for 20 min and again at 100 000 *g* for 60 min. The final pellet (membrane preparation) was resuspended in 1 ml of PBS, sonicated and stored with the supernatant at −80°C. Bradford assays were carried out on all samples to determine the protein concentration. Before use all samples were UV irradiated for 30 min and 1D3 and NEO were γ-irradiated for 13 min. All membrane preparations were added to the PBMC in triplicate at two dilutions, 5 μg/ml and 50 μg/ml (final concentration).

Culture conditions

PBMC were prepared by Ficoll–Hypaque density gradient centrifugation and the cells were subsequently cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin 100 U/ml and streptomycin 100 μg/ml (all from Flow, Irvine, UK). PBMC for the experiments involving membrane proteins and for the interferon-gamma (IFN-γ) assay were $CD8^+$ cell-depleted by incubation with OKT8 MoAb and magnetic beads coated with anti-mouse IgG (Dynal, Oslo, Norway) as described elsewhere [17].

Short term T cell lines were produced by the method of Roep *et al.* [18] to examine proliferative responses to the eye muscle membrane protein 1D3. Briefly, 10^6 PBMC per well of a 12-well culture plate were cultured with 40 μg/ml of 1D3. After 5 days, rIL-2 (25 U/ml; Boehringer Mannheim, Lewes, UK) was added and the cells cultured for a further 5 days. On day 10, 10^6 autologous PBMC (γ-irradiated) were added per well with 1D3 (200 μg/ml) and phytohaemagglutinin (PHA; 1 μg/ml). After 3 days, rIL-2 (50 U/ml) was added and the cell lines expanded over a further 4 days before being assayed. Autologous PBMC (10^6; γ-irradiated) were added to each well, as antigen-presenting cells, and the cells were harvested and washed before assay. Eight cell lines were produced, four from each of two patients.

T cell proliferation assays

The same procedure was used to test the proliferative responses of T cells to the four groups of antigen under study (eluted eye muscle antigen, antigen from nitrocellulose filters, fusion proteins and eye muscle/CHO membrane antigens). PBMC were cultured at a concentration of 10^5 cells/well (final volume 200 μl) in 96-well flat-bottomed plates, and the test antigen or control was added in replicate at the concentrations indicated below. To one replicate set of cells, PHA was added at 1 μg/ml as a positive control to ensure the PBMC were capable of proliferation. The cells were cultured for 5 days and, 16 h before termination, 1 μCi of ^{3}H-thymidine was added to each well. Thymidine incorporation was measured by harvesting onto glassfibre filters and liquid scintillation counting. Results were expressed as a stimulation index (SI):

$$SI = \frac{\text{ct/min produced by test antigen}}{\text{ct/min produced by control}}$$

ELISPOT assay

The production of IFN-γ by antigen-stimulated T cells was assessed with $CD8^+$ cell-depleted PBMC populations from six Graves' patients and two controls, according to the method of Czerkinsky *et al.* [19], as an alternative method of assessing T cell responses to orbital antigens. Briefly, 2×10^5 cells/well were added to 96-well nitrocellulose-bottomed plates coated with a MoAb to human IFN-γ (Quadratech, Epsom, UK) and incubated with the eye muscle/CHO membrane preparations, described in the previous section, for 48 h at 37°C. After washing the plate, 2 μg/ml of a biotinylated MoAb to human IFN-γ (Quadratech) were added for 3 h at room temperature. After further washing, 100 μl/well of the Vectastain ABC complex (Vector Labs, Burlingame, CA) were added for 1 h at room temperature. The plate was given a final wash (×2) and the substrate (20 mg 3-amido-9-ethyl carbazole in 2·5 ml DMF and 47·5 ml 50 mM sodium acetate pH 5 with 25 μl 30% H_2O_2) was added. After 3–5 min the reaction was terminated by running the plate under tap water. The brownish spots indicating IFN-γ-secreting cells were enumerated under low magnification using a stereo microscope.

Statistical analysis

A stimulation index of greater than 2·0 was regarded as a positive response. The frequency of responses between the groups was compared by Fisher's exact test.

RESULTS

Eluted HEM antigens

The T cell responses to the 20 protein fractions obtained by electro-elution of a 100 000 *g* HEM preparation showed no disease-specific proliferative pattern. Five of the 10 patients with TAO showed weakly positive responses (SI > 2·0) to one or more protein fraction (Table 2). Fractions of around 95 kD stimulated T cells from controls and patients with Graves' disease, irrespective of TAO; it is possible that antigen(s) of this weight may have contaminated adjacent wells, or in some elutions have been assigned a slightly different size to account for the responses seen with fractions of around 90 and 100 kD. Two of the TAO patients responded to eluted antigens of lower molecular weight, and these fractions failed to produce stimulation of any control PBMC. However, there was no significant difference between the T cell responses of TAO patients compared with those of Graves' patients without eye disease and normal controls (combined) for any of the fractions studied, nor between the entire group of Graves' patients (with or without TAO) compared with the controls ($P > 0·05$).

Solubilized recombinant proteins from nitrocellulose filters

None of the PBMC preparations from the Graves' patients without TAO ($n = 10$) or from the normal controls ($n = 7$) was stimulated by the R1 and R14 recombinant eye muscle proteins. One of the seven patients with TAO showed a weakly positive T cell response (SI = 2·1) to R1, but none responded to

Table 2. Stimulation indices (SI) produced by fraction of gel-eluted human eye muscle membranes in T cell proliferation assays. Only results with SI > 2·0 are shown

Group	Molecular weight of fraction (kD)	SI
TAO ($n = 10$)		
Patient 1	90, 95, 100	2·5–5·6
Patient 2	95	2·0
Patient 3	60, 50, 45, 35, 25	2·1–3·3
Patient 4	95, 40	2·4
Patient 5	100	4·1
Graves' disease without TAO ($n = 3$)		
Patient 1	95	2·2
Normal subjects ($n = 5$)		
Subject 1	120, 95	2·1–4·3
Subject 2	95	2·4
Subject 3	95	3·3
Subject 4	90	2·2

TAO, Thyroid-associated ophthalmopathy.

R14. The mean ± s.d. SIs produced by R1 were 1·3 ± 0·5 (TAO group), 1.1 ± 0.3 (Graves' disease without TAO) and 1·0 ± 0·2 (controls); the respective SIs with R14 were 1·0 ± 0·3, 0·9 ± 0·2 and 0·8 ± 0·2. Comparisons of the response of TAO patients and non-TAO patients (Graves' and normals) showed no statistically significant differences ($P > 0·05$).

Purified recombinant proteins

The T cell responses to D1 and R1 at the two concentrations used were similar (Table 3). One of eight patients with TAO and one of eight normal controls showed a weak positive response to D1, whereas PBMC from seven Graves' patients without TAO failed to proliferate with this antigen. When the response of patients with TAO was compared with subjects without TAO (Graves' disease and/or controls) there was no significant difference ($P > 0·05$). None of the patients tested showed a positive T cell response to the eye muscle protein R1.

Eye muscle and CHO membrane antigens

Using undepleted PBMC, one of eight patients with TAO responded weakly to both HEM membrane (SI 3·1) and supernatant preparations (SI 2·6), and also to pig eye muscle (SI 3·2) and pig skeletal muscle (SI 3·0). Two out of six normal controls responded weakly to HEM membrane (SI 2·4 and 2·6), one to HEM supernatant (SI 2·4) and two of the three Graves' patients without clinical TAO reacted to HEM membrane (SI 2·2 and 2·1). When the same experiments were carried out using CD8-depleted PBMC, three patients with TAO responded to HEM membrane, but so did two of three Graves' patients without TAO, and one of six controls (Table 4). Six of the seven subjects whose lymphocytes were stimulated by HEM were also stimulated by porcine eye muscle, leaving one patient who responded exclusively to HEM membrane (SI 5·1) and to HEM supernatant (SI 15·5).

The response of either the intact or the $CD8^+$-depleted T cell populations to the CHO eye muscle membrane antigen 1D3 was limited. Two of the eight TAO patients studied had positive responses (SI 3·1 and 5·3), but one of the patients also responded (SI 4·1) to NEO, the control membrane antigen. One of the six normal controls responded to 1D3, but again showed reactivity to NEO (SI 4·3 and 7·3, respectively). Using $CD8^+$ T cell-depleted PBMC, there were no responses to 1D3 (SI range 0·4–1·1 for TAO patients, 0·9–1·0 for controls).

Short term T cell lines

PBMC from two TAO patients were used to produce eight short term T cell lines. Only three lines from one patient showed positive responses to the test antigens. One responded to HEM supernatant (SI 2·2), one to porcine eye muscle (SI 2·8) and the third to 1D3 (SI 3·1).

IFN-γ ELISPOT assay

$CD8^+$-depleted T cells showed no IFN-γ production above background when stimulated with either human eye muscle membranes or with CHO membranes expressing 1D3 at the same concentration as used in the proliferation assays (data not shown). PHA was used as a positive control to demonstrate that IFN-γ production could be detected. The number of IFN-γ-secreting cells detected after stimulation with PHA (1 μg/ml) ranged from 25 to $90/10^4$ total cells added.

DISCUSSION

Understanding the pathogenesis of TAO is hampered by the lack of an animal model, uncertainty over the nature of the autoantigenic target(s) and the difficulty of studying *in vitro* the T cells infiltrating the eye muscles, which are likely to contain the autoreactive population. As a result, resort has generally been made to the circulating T cell population, although this is unlikely to contain the same frequency of responding cells as the extraocular muscles. Nonetheless, if there is a cross-reactive antigen in both thyroid and eye muscle, blood-borne T cells might be expected to respond to such an antigen, as thyroid

Table 3. Stimulation indices (SI) (mean ± s.d.) produced by D1 and R1 fusion proteins in T cell proliferation assays (ranges in parentheses)

	D1		R1	
	2 μg/ml	4 μg/ml	2 μg/ml	4 μg/ml
TAO ($n = 8$)	1·1 ± 0·2 (0·7–1.4)	1·1 ± 0·6 (0·3–2·1)	1·1 ± 0·4 (0·4–1·6)	0·8 ± 0·5 (0·5–1·4)
Graves' disease without TAO ($n = 7$)	1·00 ± 0·3 (0·5–1·4)	1·2 ± 0·3 (0·8–1·5)	1·0 ± 0·6 (0.3–1.9)	1·0 ± 0·4 (0·6–1·5)
Normals ($n = 8$)	1·1 ± 0·6 (0·4–2·3)	0·9 ± 0·5 (0·2–1·5)	1·2 ± 0·3 (0·7–1·6)	0·8 ± 0·4 (0·3–1·5)

TAO, Thyroid-associated ophthalmopathy.

Table 4. Stimulation indices (SI) produced by muscle membranes and supernatant and recombinant fusion protein 1D3 in T cell proliferation assays using CD8-depleted lymphocytes

Group	SI in response to antigen				
	HEM membrane	HEM SN	PEM	PSM	1D3
TAO ($n = 8$)					
Patient 1	2·2 (0·5)	1·9 (0·9)	4·3 (0·9)	1·9 (0·6)	1·1 (0·7)
Patient 2	5·1 (0·9)	15·5 (0·9)	0·8 (0·9)	1·2 (0·7)	0·4 (1·0)
Patient 3	2·3 (0·1)	2·3 (0·6)	3·5 (0·8)	2·9 (1·3)	1·3 (0·9)
Graves' disease without TAO ($n = 3$)					
Patient 1	4·3 (2·2)	4·4 (1·3)	3·1 (1·4)	1·6 (0·8)	0·9 (0·6)
Patient 2	3·3 (2·1)	1·9 (1·2)	2·8 (1·8)	2·3 (1·8)	1·6 (0·7)
Normal subjects ($n = 6$)					
Subject 1	2·2 (1·1)	1·2 (1·0)	2·1 (0·1)	1·4 (0·1)	0·9 (0·5)

HEM, Human eye muscle; SN, supernatant; PEM, porcine eye muscle; PSM, porcine skeletal muscle.

Figures in parentheses give SI before CD8 cell depletion. Only results with SI > 2·0 are shown.

antigen-specific T cells can be identified (albeit at low level) in the circulation, particularly after removal of the non-specific suppressor influence of the $CD8^+$ subset [17,20,21]. Furthermore, an increase in soluble IL-2 receptor levels and a decrease in $CD8^+$ T cell numbers can be detected in the circulation of patients with severe TAO [22,23], indicating that the ostensibly localized process in ophthalmopathy can produce detectable effects in the blood.

There have been few functional studies of T cells in TAO, and these have mainly used the migration inhibition factor assay, with contradictory results, at least in part due to the heterogeneous nature of the antigen preparations used to induce T cell stimulation (reviewed in [24]). We have previously shown that a proportion of patients with thyroid disease, irrespective of the presence of clinical TAO, have circulating T cells that proliferate in response to HEM membranes [25]. These preparations will contain antigens derived from eye muscle cells, fibroblasts and other sources. Initial characterization of the antigen was performed by fractionating the HEM by gel electrophoresis, and transferring it to nitrocellulose, which was rendered particulate [16] and then used to stimulate PBMC. Fractions of around 72–78, 52–56 and 12 kD induced weak proliferation in four of nine patients with thyroid disease, irrespective of the presence of TAO [25].

We have now extended these studies to examine a larger group of patients using gel-eluted antigen, which may be preferable to antigen bound to nitrocellulose, as the latter can induce non-specific proliferation (unpublished observations, [15]). We have also examined the response of TAO PBMC to recombinant antigens which have been isolated by screening an eye muscle cDNA library with sera from patients with thyroid autoimmunity [13], and are thus candidates for a cross-reactive antigen between thyroid and orbit. These included 1D3, a 64-kD protein, and D1, a 97 amino acid fragment of this [26], which have previously been suggested to be of relevance to TAO pathogenesis.

Our results show that the frequency of circulating T cells in TAO reacting with the antigens detailed is very low. Using eluted HEM antigen, PBMC proliferation was observed in patients and controls with fractions of around 90–100 kD. However, two of the 10 TAO patients responded to lower molecular weight fractions, including a protein of 50 kD; this may include the previously identified fraction of 52–56 kD [25], as a precise estimate of the apparent molecular weight is difficult. Alternatively, the different sizes of antigen recognized by the T cells in the two studies could reflect the heterogeneity which seems to exist between patients with this condition. Late in disease many orbital antigens may be recognized by T cells which do not play a role in the initiation of TAO. Responses were not observed with fractions of around 64 kD, suggesting that HEM antigens of this size do not cause T cell stimulation. Intact HEM membranes stimulated PBMC from TAO and Graves' patients, particularly after $CD8^+$ T cell depletion, but one of six controls also responded. The antigen responsible may also be present in porcine EM, as there was stimulation in most of these PBMC preparations with this antigen. The whole HEM supernatant produced sporadic responses, the results suggesting that the antigen is not exclusive to membrane.

Recombinant proteins identified by screening orbital cDNA libraries with sera from patients with autoimmune thyroid disease, including the protein 1D3 and D1 fragments, also failed to stimulate T cells specifically, even after removing $CD8^+$ cells. These negative results were obtained using solubilized recombinant proteins and transfected CHO cell membranes, and the latter experiments suggest that the positive proliferative responses in some TAO patients with HEM antigens are not due to the 64-kD protein 1D3. Purified recombinant 1D3 stimulated one TAO and one control PBMC preparation; this is compatible with the presence of autoantibodies to this protein in normal subjects and patients with autoimmune thyroid disease [27]. Our findings are in contrast to preliminary results indicating that T cell responses

to D1 are found in a third of TAO patients but are absent in normal subjects [12]. We also confirmed the low frequency of orbital antigen-sensitized T cells using the IFN-γ ELISPOT assay, which quantifies the number of T cells responding to an antigen [19]. No specific T cell responses were identified using this method.

In conclusion, sporadic T cell proliferative responses to eye muscle proteins can be detected in patients with TAO, but the response is weak and heterogeneous. T cell reactivity against the 64-kD protein 1D3 is not associated with the presence of TAO. These results suggest that the frequency of eye muscle-reactive T cells in the circulation of patients with TAO is low, and therefore determining the nature of the autoantigen recognized by T cells in TAO will be difficult, although the use of dendritic cells to present antigens may be one way responses could be improved [28]. An alternative approach may be to expand non-specifically the few infiltrating T cells which can be obtained from orbital biopsy specimens, as shown recently in an analysis of T cell cytokine production in TAO [29]. These cells could then be tested for reactivity to specific antigens.

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