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Immunohistochemical analysis of the retrobulbar tissues in Graves' ophthalmopathy

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SUMMARY

We have characterized the mononuclear cell infiltrate in the extraocular muscle of three patients with Graves' disease, using antibodies which permit staining of paraffin-embedded tissue. The majority of lymphocytes, occurring in foci or interstitially, were T cells, most of which stain for CD3 or with UCHL1. T cells few, if any, stained with SN130, directed against the CD45R determinant. This suggests that these lymphocytes comprise a recently activated population within which memory cells may reside. B cells were also found but were predominantly confined to focal aggregates, and in one patient lymphoid follicles were seen. The orbital fat and connective tissue from a further two patients contained very few infiltrating cells which were mainly UCHL1-positive. Eye muscle cells did not express Ia antigens but the interstitial cells between them were Ia-positive and the vascular endothelium in four of the five specimens also stained with Ia. These results indicate that Graves' ophthalmopathy is associated with T cell, and to a lesser extent B cell, responses against the retrobulbar tissues; the extraocular muscle interstitial cells, probably including fibroblasts, may be targets of activation resulting from this infiltration.

Keywords Graves' disease ophthalmopathy lymphocyte subsets

INTRODUCTION

There is now considerable evidence that the eye disease which accompanies hyperthyroidism is an autoimmune disorder (Jacobson & Gorman, 1984). Although severe ophthalmopathy only occurs in about 5% of Graves's disease patients (Graves' ophthalmopathy), subclinical disease can be detected in up to 90% if sensitive techniques are used to examine the eye muscles (Werner, Coleman & Franzen 1974; Enzmann, Donaldson & Kriss, 1979). Similar changes occur much less frequently in Hashimoto's thyroiditis or in the absence of clinical thyroid disease (ophthalmic Graves' disease).

The etiology and pathogenesis of Graves' ophthalmopathy are unclear, and there is no explanation for the close association between eye and thyroid disease. However, studies with high resolution CT scanning show that the earliest changes occur in the extraocular muscles, which seem to constitute the target of an autoimmune response (Trokel & Jakobiec, 1981). Better understanding of Graves' ophthalmopathy should come from studying the lymphocytes infiltrating extraocular muscle. Such material is now rare because many patients respond to medical therapy alone and, even if the eyes require surgical decompression, there is little indication for biopsy of eye muscle tissue since diagnostic tests of thyroid function have improved so much. However the recent development of monoclonal antibodies which react with a range of lymphocytic antigens in routinely-fixed, paraffin-embedded tissues now allows retrospective immunohistochemical studies on archival material. In the present study biopsies of eye muscle from three patients with Graves' ophthalmopathy obtained up to 29 years previously have been investigated with a panel of such antibodies in order to identify the nature of the mononuclear cell infiltrate. Similar studies for comparative purposes have been performed on frozen sections from retrobulbar fat and connective tissue obtained at more recent surgical decompression.

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MATERIALS AND METHODS

Patients

Sections of paraffin-embedded orbital tissue were obtained from three patients. Patient 1 was a 62-year-old man with known Graves' disease and active ophthalmopathy who suffered a windscreen injury to the cornea which led to enucleation in 1969 because of retinal detachment and intraocular haemorrhage. Patient 2 was a 53-year-old man with ophthalmic Graves' disease whose extraocular muscles were biopsied in 1969 because disease was initially unilateral. Patient 3 was a 44-year-

old woman with a non-toxic goitre and ophthalmic Graves' disease who in 1959 also had a biopsy because disease was unilateral. A biopsy of the levator palpebrae in both these patients showed lymphocytic infiltration and there was no evidence, in either set of biopsies, of granulomata. Biochemical and immunological investigations were not available for historical reasons. Patients 4 and 5 were women aged 33 and 57; both had Graves' disease and severe ophthalmopathy requiring decompression. Orbital fat and connective tissue were snap-frozen in these two cases. A control paraffin-embedded specimen of normal retro-orbital tissue was obtained from a patient with an intraocular malignancy.

Immunohistochemistry

Sections from the paraffin-embedded material were dewaxed with xylene, transferred to alcohol and finally into Tris-buffered saline, pH 7·4. Trypsinization for predetermined optimal times was performed prior to using the polyclonal anti-CD3 serum and the monoclonal antibodies MAC-387 and CR3/43. Cryostat sections (5 μm) were cut from the frozen specimens after mounting in OCT medium (BDH, Poole, Dorset), mounted on poly-L-lysine coated slides and fixed in acetone. Details of the antibodies used are given in Table 1. Staining for the mouse monoclonal antibodies was completed by two to three 10-min alternate cycles of anti-mouse Ig and alkaline phosphatase monoclonal anti-alkaline phosphatase complex (APAAP) (Dakopatts a/s, Glostrup, Denmark), followed by the addition of freshly made and filtered substrate (2 mg naphthol AS-MX phosphate, 10 mg fast red, 200 μl dimethylformamide, 2·4 mg levamisole in 10 ml 0·1 M Tris buffer, pH 8·2) for 20 min (Cordell *et al.*, 1984). The polyclonal rabbit anti-CD3 serum required the insertion of a staining step with monoclonal mouse anti-rabbit immunoglobulin before proceeding to the APAAP immunostaining procedure as above (Mason *et al.*, 1988), and the staining for desmin, α-1-anti-trypsin and lysozyme was performed by the immunoperoxidase technique. Sections were lightly counterstained with haematoxylin.

RESULTS

The extraocular muscles from patients 1 and 2 contained large aggregates of lymphocytes, some surrounding degenerating muscle, as well as a scattered interstitial infiltrate. Many of the lymphocyte clusters were around or closely associated with blood vessels; no prominent aggregation was seen around nerve fibres. In both specimens there was oedema and some fibrous tissue replacement but no infiltration by fat cells. The muscle specimen from patient 3 was much smaller than the other two and contained a mild interstitial infiltrate of lymphocytes with some oedema.

The majority (about two-thirds) of the lymphocytes stained with the polyclonal anti-CD3 serum; about half the remainder stained weakly with LN1 and almost all the remainder more strongly with 4KB5 (which identifies mainly B cells). In patient 1 several clusters of CD3-positive lymphocytes were seen surrounding LN1 and 4KB5-positive cells (Fig. 1), but in patient 2 the organization of T and B cells was less clear. T cells predominated, particularly in areas of interstitial infiltration (Fig. 2). In patient 3 nearly all the infiltrating cells were CD3-positive.

Many of the T cells in patients 1 and 2 stained with UCHL1 (Fig. 3) and some of the cells in these areas also stained with SN130 (and 4KB5). Areas which stained with LN1 and 4KB5 were also stained with SN130. In general about 80% more cells stained with UCHL1 than SN130 or 4KB5 in areas identified as staining mainly with the CD3 antiserum. It seemed possible that a few cells stained with both UCHL1 and SN130 (or 4KB5) as judged by the appearances on serial sections. However, double staining of one specimen with UCHL1 and SN130, kindly performed by Dr P. Amlot (Royal Free Hospital), showed complete dichotomy of the two populations. The lymphocytes clustered around blood vessels nearly all stained with UCHL1 and/or SN130 (Figs 3 and 4). In patient 3, almost all the infiltrating cells were UCHL1-positive and about 20% were SN130-positive.

Very few cells stained with MAC-387: α-1-anti-trypsin or lysozyme-positive reactivity was almost always confined to cells

Table 1. Monoclonal and polyclonal antibodies used in this study

Antibody	Specificity	Isotype	Source	Reference
Anti-CD3	CD3 (T cells)	Polyclonal	Oxford	Mason *et al.*, (1988)
4KB5	B cells (CD45R)	IgM	Oxford	Davey *et al.*, (1987)
LN1	B cells (germinal centre)	IgM	ICN Immunobiologicals, IL, USA.	Epstein *et al.*, (1984)
MAC-387	Monocytes, some macrophages	IgG1	Dakopatts, High Wycombe, Bucks., UK.	Flavell *et al.*, (1987)
Anti-lysozyme	Macrophages	Polyclonal	Dakopatts, High Wycombe.	
Anti-alpha-1-anti-trypsin	Macrophages	Polyclonal	Dakopatts, High Wycombe.	
UCHL1*	T cell subset, monocytes granulocytes	IgG2a	Dr P. Beverley	Smith *et al.*, (1986)
SN130	Putative CD45R	IgG1	Dr P. Amlot	Akbar *et al.*, (1988)
TAL-IB5*	HLA-D α Chain (non polymorphic)	IgG1	Dr J. Bodmer	Epenetos *et al.*, (1985)
CR3/43	HLA-DR β Chain	IgG1	Oxford	Ghosh *et al.*, (1984)
Anti-desmin	Muscle	Polyclonal	Bio-Nuclear Services, Reading, Berks., UK.	

* Available from Dakopatts.

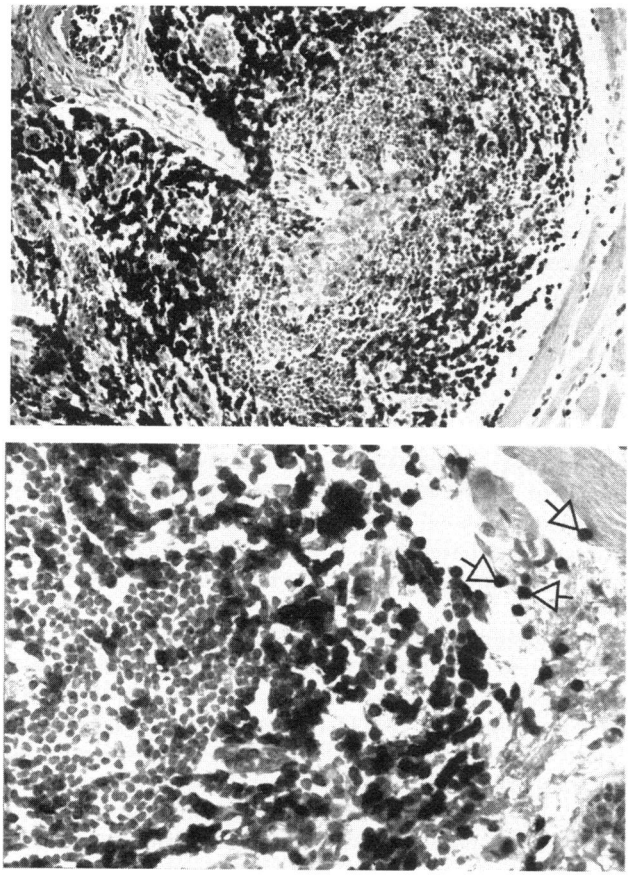

Fig. 1. CD3-positive lymphocytes in ophthalmopathy. Top—lymphoid follicle in extraocular muscle. The dark cells were stained with the polyclonal anti-CD3 serum; the unstained cells in the centre were 4KB5-positive (i.e. B cells) in a separate section (Original magnification × 400). Bottom—high power view showing CD3-positive cells surrounding unstained cells to the left of the picture; scattered CD3-positive cells are also seen between muscle cells (arrowed) (Original magnification × 800).

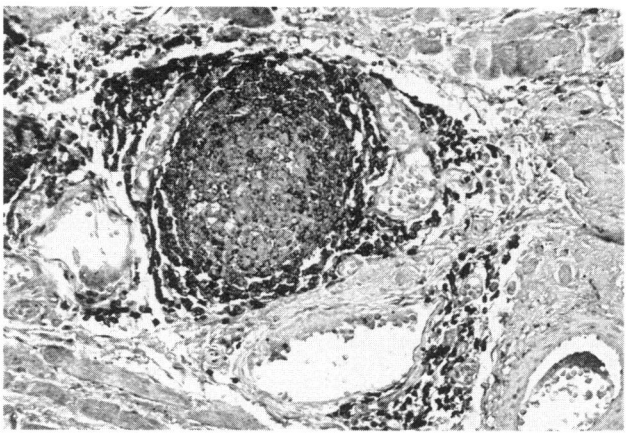

Fig. 3. Extraocular muscle lymphocytic infiltration in association with blood vessels, stained with UCHL1 (Original magnification × 400).

within blood vessels. With TAL-IB5 (which was quite weak) and CR3/43 (both against Ia) staining was seen in the B cell regions identified in patient 1, and about 25–50% of the remaining lymphocytes in foci or interstitially were positive for staining in patients 1 and 2. Very few lymphocytes were positive in patient 3. Many of the predominantly T cell areas showed only a few Ia-positive cells (Fig. 5). There were scattered Ia-positive cells with the morphology of macrophages, and blood vessel endothelium was strongly positive for CR3/43 in patient 1, with patchy positivity in patients 2 and 3. The muscle cells, identified by staining for desmin, were always negative for Ia stained on a serial section to the desmin. However in patients 1 and 3 many of the interstitial cells, lying in the connective tissue between the muscle fibres, were positive for staining with CR3/43, but not TAL-IB5 (Fig. 5). These cells were large and flattened, and were negative for staining with desmin, MAC-387, α-1-anti-trypsin and lysozyme.

The orbital connective tissue from patients 4 and 5 revealed very little abnormality: a single small focus of lymphocytes (UCHL1- and SN130-positive in a ratio of about 2:1) was seen in the material from patient 4 and only a few UCHL1-positive, SN130-negative cells were seen in patient 5. In both cases the

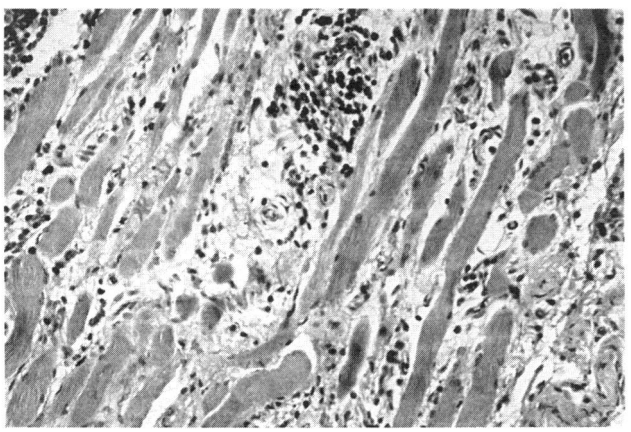

Fig. 2. Extraocular muscle focal and interstitial lymphocytic infiltrate stained with anti-CD3 (Original magnification × 400).

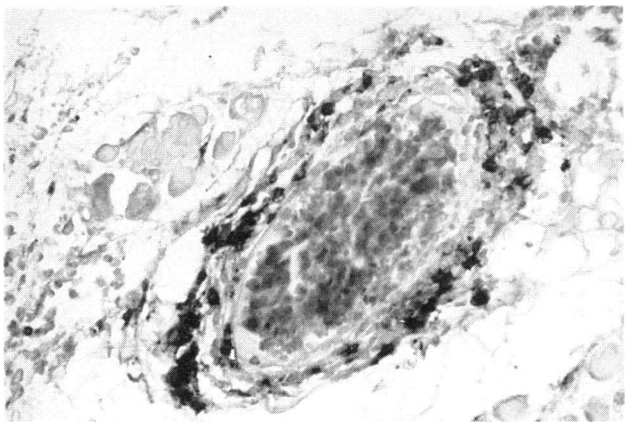

Fig. 4. Perivascular lymphocytic infiltration stained with SN130 (Original magnification × 800).

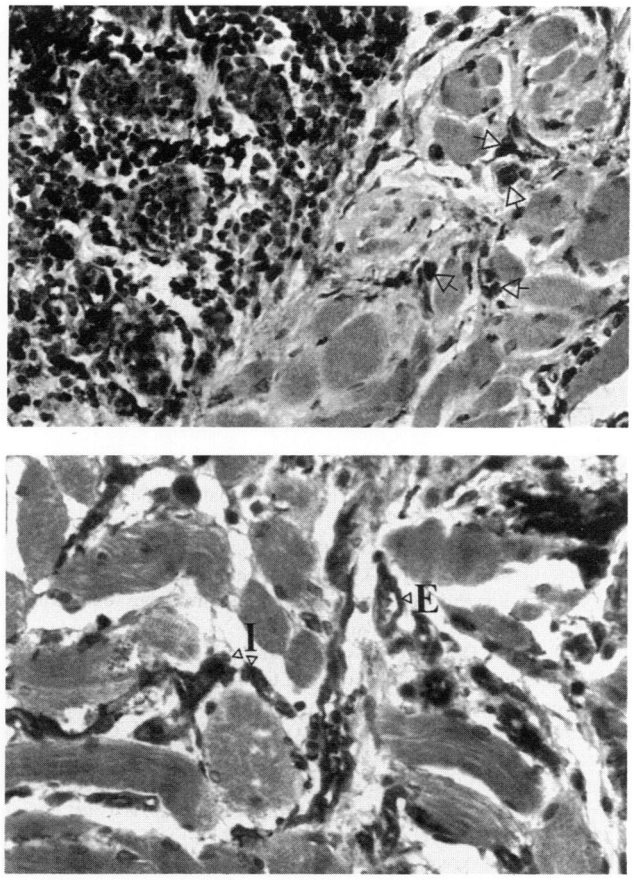

Fig. 5. Extraocular muscle stained with CR3/43. Top—a few infiltrating lymphocytes were stained: muscle fibres did not stain with CR3/43 but most of the interstitial cells were positive (arrows) (Original magnification ×400). Bottom—high power view showing positive staining of capillary endothelium (E) as well as elongated interstitial cells (I) (Original magnification × 800).

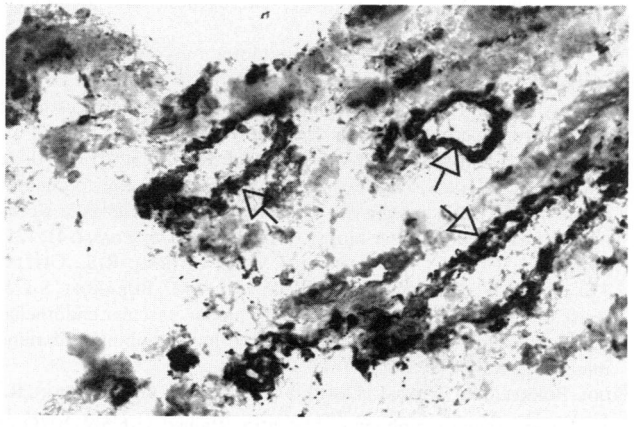

Fig. 6. Orbital connective tissue stained with TAL-IB5. The blood vessel endothelial cells are positive (arrows) (Original magnification × 400).

endothelium was TAL-IB5- and CR3/43-positive (Fig. 6). The control muscle and orbital tissue showed no lymphocytic infiltration and also failed to stain with TAL-IB5 or CR3/43.

DISCUSSION

The morphological features in these patients largely agree with previous reports of Graves' ophthalmopathy (Kroll, 1966; Jellinek, 1969; Trokel & Jakobiec, 1987; Campbell, 1984). The predominant features in the extraocular muscles of the three patients studied here were of a diffuse and focal lymphocytic infiltrate, oedema and some fibrosis. In patients 1 and 2 there was a prominent association of lymphocytic aggregates with blood vessels, a finding only highlighted in one previous report (Jellinek, 1969). Fat cell infiltration of the extraocular muscles, which can be very pronounced in ophthalmopathy and which seems to increase with disease duration (Daicker, 1979), was not found in our cases. In contrast to the changes in the eye muscles, the orbital fat and connective tissue in patients 4 and 5 contained very few lymphocytes (and the retro-orbital tissues were reported as histologically normal in patient 1 at the time of enucleation). This lack of lymphocytic infiltration has also been observed by others (Tengroth, 1964; Trokel & Jakobiec, 1981).

Because CT scanning and serological testing can now readily establish the diagnosis of Graves' ophthalmopathy, biopsies of extraocular muscle are rarely available for frozen section immunohistochemical studies. However use of archival material and antibodies which recognize epitopes in paraffin-embedded sections has allowed us to perform partial characterization of the lymphocytic infiltrate. The majority of lymphocytes were T cells, which stained with UCHL1 (Smith *et al.*, 1986). Although UCHL1 staining predominated, it is possible that some cells were positive for both UCHL1 and CD45R markers. However no such cells were found in one specimen in which double staining was performed. Recently it has been established that phytohaemagglutinin stimulation *in vitro* causes a unidirectional change in T lymphocyte phenotype from CD45R-positive to UCHL1-positive cells, with a transitional phase of double positivity; antigen-specific proliferating cells and antigen-specific cytotoxic cells are greatly enriched in the UCHL1-positive T cell subset (Akbar *et al.*, 1988). Thus the T lymphocytes in eye muscle appear to contain a major population of recently activated and memory cells.

Although B cells were also prominent in patients 1 and 2, staining with 4KB5, SN130 and to a lesser extent LN1, they were outnumbered by T cells identified with the polyclonal CD3 antiserum and were usually found in foci; in patient 1 some of these foci had the appearance of true follicles. There were very few cells positive for staining with the macrophage markers, MAC-387, α-1-anti-trypsin and lysozyme. The majority of positive cells were within blood vessels, although lymphocytic aggregates contained rare cells staining with α-1-anti-trypsin and lysozyme.

Eye muscle cells were negative for Ia staining, although this can be induced on such cells with γ-interferon (γ-IFN) *in vitro* (Hiromatsu *et al.*, 1987). This is of some interest, since aberrant Ia expression by the thyroid follicular cells in Graves' disease has been suggested as a possible initiating factor in the autoimmune response (Bottazzo *et al.*, 1983). The present findings would suggest that a similar mechanism is unlikely to prevail in Graves' ophthalmopathy. However interstitial cells

were Ia-positive in patients 1 and 3. The exact nature of these cells could not be identified in the present study, in the absence of a suitable marker for fibroblasts, but they did not appear to be lymphocytes, macrophages or muscle cells on staining criteria. At least some would appear to be fibroblasts, given the frequency of Ia-positivity and their location; some may also have been mast cells.

This aberrant Ia expression possibly results from the local release of T cell-derived lymphokines like γ-IFN (Parker *et al.*, 1983; Hiromatsu *et al.*, 1987), and is compatible with the hypothesis that a major pathogenetic mechanism in Graves' ophthalmopathy is stimulation of extraocular muscle fibroblasts by infiltrating lymphocytes (Trokel & Jakobiec, 1981; Campbell, 1984). It is not clear why the muscle cells were not Ia-positive, assuming that other cells became positive as a result of lymphokine release. It is possible that other factors, besides γ-IFN, are needed to induce muscle cell Ia *in vivo* in contrast to the situation *in vitro* (Hiromatsu *et al.*, 1987). Local differences in specific cell sensitivity to the effects of lymphokines on Ia induction have also been found in the pancreas in diabetes (Pujol-Borrell *et al.*, 1987). Vascular endothelium was also Ia-positive in the extraocular muscle and in the orbital connective tissue of all five patients. This latter finding is similar to the endothelial Ia-positivity around the islets in diabetes mellitus (Bottazzo *et al.*, 1986) and occurring in the thyroids of rats with experimental autoimmune thyroiditis (Cohen, Dijkstra & Weetman, 1988). A role for this phenomenon in disease has not yet been elucidated but could include effects on lymphocyte homing.

In summary, these findings confirm that the extraocular muscles are the main site of the autoimmune process in Graves' ophthalmopathy. The infiltrate in these muscles from three patients with Graves' ophthalmopathy consisted predominantly of T cells, and to a lesser extent B cells. The T cells appeared to be recently activated, memory cells, as judged by staining with UCHL1. Eye muscle Ia expression was not found but cells in the interstitial connective tissue were Ia-positive in two of three cases. This is in keeping with the idea that activation of these cells could have a role in the pathogenesis of ophthalmopathy.

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