Extraocular muscle biopsies from normal individuals and five patients with Graves' ophthalmopathy were analyzed by immunohistochemical staining. Fibroblasts in normal extraocular muscle as well as Graves' extraocular muscles expressed HLA-class II antigens, but the muscle cells did not. There was an increase of interstitial tissue in Graves' extraocular muscles but no visible damage to the muscle cells. In four of the biopsies from Graves' patients the cellular reaction was very weak. In the fifth patient the reaction was more pronounced with macrophages predominating, but with few lymphocytes and almost equal amounts of B and T cells. We could not confirm the earlier described specific extraocular muscle antibodies in sera from Graves' patients and conclude that there seems to be an activation of orbital fibroblasts in Graves' ophthalmopathy. This may contribute to the pathogenesis of the disease. Invest Ophthalmol Vis Sci 29:175-184, 1988

Graves' ophthalmopathy is a disorder of still unknown origin. In an animal model thyrotropin (TSH) has been shown to produce exophthalmos and in vitro studies have demonstrated binding of TSH and an "exophthalmogenic factor" to orbital tissues. Findings have also indicated binding of thyroglobulin-antithyroglobulin immune complexes to extraocular muscle membranes. There has been evidence of cell mediated immunity against retrobulbar tissue and more recently, findings implicating the existence of circulating antibodies directed against eye muscle antigens.

Several investigators have studied the pathology of orbital tissue in Graves' ophthalmopathy and most authors agree on the character of the changes. In brief, there are interstitial edema and infiltrating macrophages, lymphocytes, mast cells and plasma cells between the muscle fibers, but no evidence of primary muscle cell damage. The reaction seems to take place in the interstitial tissue and causes an increase of the ground substance and collagen production.

The present study was done to further characterize the cell population in the extraocular muscle in Graves' ophthalmopathy, using a panel of monoclonal antibodies against various cell surface antigens. We have also tried to demonstrate circulating antibodies against extraocular muscle antigens in Graves' ophthalmopathy patients using Western blot technique (WB) and indirect immunofluorescence (IFL).

Materials and Methods

Patients

Extraocular muscle biopsies were taken from five patients with Graves' ophthalmopathy. All five patients gave their informed consent to the biopsies.

Case 1: A 47-year-old woman was found to have hyperthyroidism in March 1984 and propylthiouracil was given. A few weeks later she started to feel discomfort in her eyes and eyelid edema and chemosis were noted. Six months later thyroxin was added and in December she developed bilateral exophthalmos, which slowly progressed during the following 6 months. In June 1985 a subtotal thyroidectomy was performed. During this operation a transconjunctival biopsy was taken from the right lateral rectus muscle, approximately 1.5 cm from its insertion. The muscle was moderately swollen at gross examination. The patient was not on steroid medication.

Case 2: A 39-year-old woman in whom hyperthyroidism and ophthalmopathy were diagnosed in 1970. Carbimazol and thyroxin were administered during 3 years which resulted in an improvement of the eye signs. In 1978 the ophthalmopathy relapsed and 131I was given. Three years later there was a new...
relapse and corticosteroids were given. Because of failing patient compliance retrobulbar irradiation was administered in April 1982 and steroids were withdrawn. In 1985 the ophthalmopathy progressed and a transconjunctival biopsy was taken from the right medial rectus muscle, which appeared red and swollen. At this time the patient had a marked bilateral proptosis, lid retraction and obvious injection over the extraocular muscles.

Case 3: A 64-year-old woman with hyperthyroidism diagnosed in 1981 and treated with carbimazol. Shortly after treatment was instituted she developed ophthalmopathy with slowly progressive exophthalmos predominantly of the left eye, keratitis and later restriction of upward gaze and a recession of the left inferior rectus muscle was made. In March 1983 the patient was given 131I and 8 months later her ophthalmopathy progressed. There were marked congestive signs in both eyes, progressive bilateral exophthalmos, bilateral restriction of upward gaze and slight bilateral optic disc edema. The ophthalmopathy did not respond to prednisolone, necessitating an orbital decompression in February 1984. During this operation a biopsy was taken from the right inferior rectus muscle, which appeared indurated. At the time of operation the patient was taking 40 mg prednisolone daily.

Case 4: A 30-year-old woman with Graves' disease diagnosed in August 1982. At this time she had slight exophthalmos and moderate congestive signs. She was treated with propylthiouracil and her eye symptoms slowly improved. Her medication was continued at a high dose but in 1985 she relapsed and had a radioactive iodine uptake of 44%. In June a subtotal thyroidectomy was performed. One month later she developed progressive exophthalmos with minor visual field defects. Steroid therapy was initiated, but the ophthalmopathy did not respond and in August 1986 an orbital decompression was made and biopsies were taken from moderately swollen inferior rectus muscles. At the time of operation the patient was receiving 40 mg prednisolone daily.

Case 5: 61-year-old woman. In January 1985 hyperthyroidism was diagnosed and the patient was treated with 131I. Shortly after treatment she developed ophthalmopathy with marked congestive signs, exophthalmos and corneal exposure. In June corticosteroids were given with only moderate effect and the steroids were completely withdrawn 4 months later. The ophthalmopathy worsened slowly and in February 1986 bilateral visual field defects were noted. Surgical decompression of both orbits was performed 3 months later. During this operation biopsies were taken from both inferior rectus muscles, which appeared markedly swollen.

Controls

Five different inferior oblique muscles, obtained from squint surgery, and one rectus muscle obtained from enucleation because of malignant melanoma, were used as controls. Skeletal muscle was obtained from mammary cancer surgery (pectoralis muscle) and from neck muscle in thyroid surgery (patient without ophthalmopathy).

Serum Samples

Sera were collected from 24 patients with hyperthyroidism and ophthalmopathy (American Thyroid Association, classes 2-6) and used in WB and IFL. Twenty-three of the patients had an active ophthalmopathy and one had inactive, "burnt-out" disease. Nine of the patients with active disease had high levels of thyroid-stimulating antibodies (TSAb). In 14, the TSAb values were within normal range. Control sera were obtained from ten patients with hypothyroidism and seven patients who had various auto-antibodies but no demonstrable thyroid disorder. Eight sera from healthy blood donors were used in the IFL and 20 in the WB. Sera were stored at -20°C.

Histochemical Staining

The biopsies were frozen within 2 hr and stored at -70°C. Four μm cryostat sections were made and stored at -70°C until use. Sections from all patients and controls were stained with hematoxylin-eosin and van Gieson.

Immunohistochemical Staining Technique

Sections were incubated with a panel of monoclonal antibodies for 30 min (Table 1). After washings, the sections were incubated with alkaline-phosphatase-conjugated (AP) rabbit anti-mouse antibodies (DAKOPATTS, Copenhagen, Denmark) for 30 min, and then with swine anti-rabbit AP-conjugated antibodies (DAKOPATTS) for 30 min. The AP Substrate Kit SK-5100 (Vector Laboratories, Burlingame, CA) was used and sections were counterstained with Harris hematoxylin (Merck, Darmstadt, West Germany).

Double staining technique with peroxidase-labeled rabbit antibodies to factor VIII-related antigen (DAKOPATTS) together with mouse antibodies to HLA-DR, detected by AP-conjugated rabbit anti-mouse antibodies (DAKOPATTS), was used in some experiments.

Mouse monoclonals together with rabbit anti-HLA-DR (gift from Dr. Lars Klareskog, Uppsala,
No. 2 IMMUNOHISTOCHEMICAL STAINING OF EXTRAOCULAR MUSCLES / Tällsred and Norberg 177

Sweden) were used in indirect IFL with double staining technique in which the rabbit antibodies were visualized by TRITC-conjugated swine anti-rabbit antibodies (DAKOPATTS) further absorbed with mouse serum. The mouse monoclonals were demonstrated by FITC-conjugated sheep anti-mouse antibodies (National Laboratory of Bacteriology, Stockholm, Sweden, nr SM 111610) absorbed with human and rabbit sera.

In all experiments negative controls consisting of normal mouse or rabbit serum and the appropriate conjugates were included. The appropriate reactions of the monoclonal antibodies were controlled using sections of human tonsils.

Determination of Antibodies Against Extraocular Muscle Antigen

For WB analysis, antigen was prepared from human extraocular muscle, obtained from squint surgery. A soluble fraction was prepared as previously described and the protein content was adjusted by the method of Lowry to 2 mg/ml. Samples were subjected to sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli and followed by Western blot. Patients' sera were diluted 1:20 and reacting antibodies were detected by horseradish peroxidase conjugated rabbit anti-human immunoglobulins (DAKOPATTS). For indirect IFL, 4 μm sections of human extraocular muscle obtained from squint surgery were fixed in cold acetone for 10 min, incubated with patients' serum (dilution 1:6) for 20 min, and FITC-conjugated sheep anti-human gammaglobulins (National Laboratory of Bacteriology, nr SH 088603-1) for 20 min.

Results

Control Muscles

Hematoxylin-eosin staining showed fibroblast-like cells in the interstitial tissue of the extraocular mus-

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
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<tr>
<td>B1*</td>
<td>B lymphocytes</td>
</tr>
<tr>
<td>OKT 11†</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>Anti-Leu-4‡</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>Anti-Leu-21§</td>
<td>T cytotoxic/suppressor lymphocytes</td>
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<tr>
<td>Anti-Leu-3§</td>
<td>T helper/inducer lymphocytes</td>
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<td>Anti-Leu-10‡</td>
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<tr>
<td>Anti-Leu-M5§</td>
<td>macrophages/macocytes</td>
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<tr>
<td>Factor VIII-related</td>
<td>capillary endothelial cells</td>
</tr>
<tr>
<td>Anti-Interleukin-2 receptor DAKO-LC§</td>
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</tr>
<tr>
<td>OKT 6†</td>
<td>leucocyte common antigen</td>
</tr>
<tr>
<td>S 100 (polyclonal)§</td>
<td>thymocytes, Langerhans cells</td>
</tr>
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<td>interdigitating dendritic cells</td>
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<td></td>
<td>glial cells, dendritic cells</td>
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* Coulter Clone, Luton, England.
† Ortho Diagnostics, Raritan, NJ.
‡ Becton-Dickinson, Mountain View, CA.
§ DAKOPATTS, Copenhagen, Denmark.

Fig. 1. Normal extraocular muscle stained with antibodies against macrophages. Arrows indicating some positive cells (original magnification ×110).
icles and van Gieson staining demonstrated numerous collagen fibers between the muscle cells. On the other hand, very few fibroblast-like cells were present in skeletal muscles and only a few collagen fibers were visualized.

Neither T lymphocytes (OKT 11 or Leu-4 positive cells) nor B lymphocytes (B 1 positive cells) were seen in control extraocular muscles or skeletal muscles. On the other hand some macrophages (cells positive for DAKO-Macrophage, Anti-Leu-M3, Anti-Leu-M5 and DAKO-LC) were found in the extraocular muscles (Fig. 1). Cells in the tissue between the extraocular muscle fibers were HLA-class II positive (cells reacting with Anti-HLA-DR, Anti-Leu-10 and Anti-HLA-DP) (Fig. 2), as were the capillary endothelial cells (Fig. 3). This was shown using double staining technique with anti-HLA-class II antibodies and antibodies to factor VIII-related antigen, which detect the capillary endothelial cells. No reactions were obtained with antibodies identifying dendritic cells (OKT 6, S 100). The muscle cells did not react with any of the antibodies used (Table 1).
In the skeletal muscles there were some HLA-class II positive structures between the muscle cells (Fig. 4) but much less in comparison with the extraocular muscles. All HLA-class II positive cells could be characterized as capillary endothelial cells.

**Graves' Muscles**

Biopsies from the first four cases had rather similar appearance. There was a moderate increase of interstitial tissue but no visible damage to the muscle cells and no loss of striation. The cellular reaction was weak with only a few lymphocytes of B or T cell origin. The muscle cells did not stain for HLA-class II but interstitial cells were positive to the same extent as in normal extraocular muscle.

The cellular reaction of case 5 was more pronounced but still the amount of lymphocytes was low with a slight dominance of T lymphocytes (Figs. 5, 6). There was no definite preponderance of T helper/in-
ducer or T cytotoxic/suppressor cells. Many cells were positive for anti-macrophage antibodies (Fig. 7), another finding that distinguished this case from the others. Like the other biopsies and the normal extraocular muscles, the cells in the interstitial tissue stained for HLA-class II antigens (Fig. 8). The staining was more intense than in the other biopsies, which might depend primarily on the greater amount of macrophages. There was no increase of capillary endothelial cells (Fig. 9). Cells of the adjacent connective tissue in the muscle sheath were also HLA-class II positive.

**Serological Analysis**

Using Western blot, all the patients with Graves' ophthalmopathy, the control patients and healthy blood donors showed reactivity with a 55 kD protein (Fig. 10). In 12 of the 23 patients with active ophthalmopathy and in the patient with inactive disease
there was a weak reactivity with a protein of approximately 150 kD. This band was also seen in seven of the 20 healthy blood donors, in four of ten patients with hypothyroidism and in one patient in the group with other auto-antibodies. In a few WB strips there was also reactivity with some other peptides.

All sera tested in indirect immunofluorescence gave a weak to moderate fluorescence of the muscle cells and interstitial tissue. The fluorescence of the muscle cells seemed to include both cytoplasm and cell surface. In some sera tested, within all the patients groups and healthy blood donors, we could distinguish a slightly more intensive fluorescence with a similar pattern.

Discussion

There was a clear morphologic difference between the extraocular muscle and skeletal muscle. In the extraocular muscle there was a considerable amount
Fig. 10. Western blot strips showing the 55 and 150 kD reaction (a) and only the 55 kD reaction (b), seen in all sera tested.

of interstitial tissue between the muscle fibers, whereas in skeletal muscle the muscle cells were more tightly packed.

Most cells of the interstitial tissue in normal as well as Graves’ extraocular muscle displayed HLA-class II antigens on their surface. Class II antigens are regularly present on macrophages, B lymphocytes and activated T lymphocytes and serve as restriction elements in antigen presentation to T lymphocytes. Class II antigens are also identified on different non-lymphoid cells such as capillary endothelial cells, various epithelial cells, Langerhans cells of the skin and dendritic cells. Moreover, class II antigen expression can be induced on other cells, normally negative, eg mitogen-stimulated thyroid follicular cells and umbilical vein endothelial cells.

The extraocular muscles were highly vascularized, demonstrated by the reaction with antibodies to factor VIII-related antigen, which is considered to be specific for capillary endothelial cells. These cells also displayed class II antigens. The non-endothelial cells of the interstitial tissue could not be fully characterized. They were HLA-class II positive and appeared morphologically as fibroblasts and a vast amount of collagen was found in the tissue. Fibroblasts cannot be identified immunologically but the cells did not react with antibodies identifying dendritic cells and only a few cells reacted with antibodies to macrophages. Fibroblasts do not normally display class II antigens, but they can apparently express these cell surface molecules, as has been shown in cultured dermal and orbital fibroblasts treated with γ-interferon.

To our knowledge there are no studies on HLA-class II expression of the orbital tissues. There have been some reports about HLA-class II positive interstitial structures in heart muscle. Dar et al suggested that the class II positive, factor VIII-related antigen negative cells in heart muscle were dendritic cells of leucocyte origin. Rowe et al examined biopsies from skeletal muscle from normal individuals and found scattered class II positive interstitial cells, which they presumed to be macrophages.

We have examined biopsies from five Graves’ patients and did not find any signs of muscle cell damage, which confirmed earlier observations. Four of the biopsies showed weak cellular reactions with only scattered inflammatory cells. The four patients had an active ophthalmopathy when the biopsy was taken, but the disease had been longstanding and there might have been more active reactions at earlier stages. Two of the patients had ongoing steroid medication, which may have diminished the inflammatory response. Moreover, the lymphocyte infiltration may be focal and not equally distributed throughout the muscle. Thus, the biopsies might not be representative for the whole muscle. Case 5, however, had a more active reaction with predominantly macrophages, but also some lymphocytes, with almost equal amount of B and T lymphocytes. The muscle cells did not express HLA-class II antigens, neither in controls nor in Graves’ patients.

The interstitial edema seen in Graves’ extraocular
muscle is accompanied by an increase of the ground substance, predominantly hyaluronic acid.9 13 In our biopsies, the amount of HLA-class II positive cells, however, did not differ significantly from controls, except in case 5, which showed an increased number of those cells. Cells in the muscle sheath stained for class II antigens in Graves’ patients but not in controls, which indicates an activation of fibroblasts in the orbit of Graves’ ophthalmopathy.

Recently Rotella et al29 showed that some clones of thyroid stimulating antibodies (TSAb), produced by heterohybridoma technique, stimulated fibroblasts in culture to produce collagen. They also found that a high percentage of sera from Graves’ patients were positive in the same assay. Our findings, that the fibroblasts seemed to be activated, ie expressed HLA-class II antigens, also focus the interest on these cells in Graves’ ophthalmopathy.

We have failed to detect any specific circulating antibodies to extraocular muscle antigens in the 24 sera tested from patients with Graves’ ophthalmopathy in WB and IFL. Other investigators have demonstrated such antibodies in IFL8 and ELISA6,7 although Salvi et al30 pointed out difficulties in ELISA with non-specificity and high background binding. We have used both soluble fraction from human extraocular muscle and membrane fraction from guinea pig hardierian gland (data not shown). We found in WB analysis that many of the patients’ sera reacted with a 150 kD peptide but this reaction was also seen in the controls. Nor could the IFL experiments distinguish the patients with Graves’ ophthalmopathy from controls. Thus we conclude that, with the standard techniques used, it is not possible to detect specific extraocular muscle antibodies. Moreover, the biopsy findings may argue against a clinically significant immunologic reaction to the extraocular muscle antigen.

In conclusion, our investigation has indicated an activation of orbital fibroblasts. This is also supported by recent findings showing a stimulation of fibroblasts by TSAb29 and it might contribute to the pathogenesis of Graves’ ophthalmopathy.

**Key words:** Graves’ ophthalmopathy, immunohistochemical staining, HLA-class II antigens, fibroblasts, extraocular muscle antibodies

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