Site-dependent Distribution of Macrophages in Normal Human Extraocular Muscles

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Purpose. Clinical data indicate that extraocular muscles have different susceptibilities for some orbital immune disorders depending on their anatomic location. The resident immunocompetent cells may be important mediators in the local pathogenesis of such disorders so the distribution of these cells was studied in extraocular muscles obtained from normal human donors. For comparison skeletal muscles were studied.

Methods. The cell distributions were analyzed quantitatively in cryostat cross-sections subjected to a two-step immunoperoxidase method using monoclonal antibodies against T cells, B cells, macrophages and several other markers for cell differentiation or activation. The macrophage distribution was analyzed in more detail using on-line semiautomatic image analysis equipment (VIDAS, Kontron, Elektronik GmbH, Eching, Germany).

Results. Extraocular muscles contain numerous macrophages, fewer human leukocyte antigenD-related (HLA-DR) positive cells and T cells, whereas B cells are absent. The numeric density of all cell types, and macrophages in particular, is much higher in extraocular muscles than in skeletal muscles. In extraocular muscles the majority of T cells are positive for the CD8 antigen (suppressor/cytotoxic), in skeletal muscle CD4 positive T cells (helper) predominate.

Conclusions. Extraocular muscles contain many more CD8-positive cells and macrophages per square millimeter than skeletal muscles. Of all the cell types studied, only the macrophage distribution differs significantly among the normal extraocular muscles: the medial and inferior recti muscles contain about twice as many macrophages as the lateral rectus and superior oblique muscles. Their mean sizes (area) or shape distributions however, appear to be similar.


In orbital tissues several types of immunopathogenic disorders occur, such as Graves' ophthalmopathy, Wegener's granulomatous, pseudolymphoma, and non-Hodgkin lymphoma.1 In many cases systemic immune abnormalities are not demonstrable.2 Although controversial, the orbit does not seem to contain lymphatic vessels, and, furthermore, there is no convincing evidence for the presence of an orbit draining lymph node in primates.3,4,5 Because normal orbital tissues contain numerous immunocompetent cells6,7,8 it has been proposed that these local cells are of significance for the initiation of orbital immune diseases.

In Graves' ophthalmopathy the extraocular muscles are diseased, but skeletal muscles appear to be unaffected.9 Furthermore, not all extraocular muscles are diseased to the same extent. Some authors report that computed tomographic scans show that the inferior and medial recti muscles appear to be strongly...
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affected, whereas, for example, the lateral rectus and superior oblique muscles are affected to a far lesser extent.\textsuperscript{10,11} More recently it has been reported that the superior muscle group is marginally more involved.\textsuperscript{12} As local immunocompetent cells might be involved in the initiation of orbital immune disorders, the normal distribution among the different extraocular muscle types may be of importance. In the current study, the cell distributions were analyzed in frozen cross-sections of muscular tissues that were stained with well-characterized monoclonal antibodies against B cells, T cells, macrophages, human leukocyte antigen-D related (HLA-DR) and several activation markers, using a two-step immunoperoxidase method (see references 6, 7, and 8). For analysis of cell distributions, cells were quantified according to a random sampling procedure. Because macrophages in particular may be important mediators in orbital immunity\textsuperscript{7} their distribution was analyzed in more detail using semi-automatic image analysis equipment (VIDAS, Kontron Elektronik GmbH, Eching, Germany). To allow optimal inter-muscle comparison the studies were carried out on complete sets (recti and obliques) extraocular muscles obtained from each donor.

**MATERIALS AND METHODS**

**Tissues**

Human extraocular recti and oblique muscles were obtained postmortem by exenteration of the orbital contents leaving the eyelids intact. Material was obtained from patients with Alzheimer’s disease (age 84, 6 hr postmortem), cervical carcinoma (age 56, 8 hr postmortem), pneumonia (age 86, 10 hr postmortem), and cardiac arrest (age 63, 5 hr postmortem), or at surgery, from a patient exenterated for basal cell carcinoma in the eyelid (age 63) and from patients enucleated for intraocular melanoma (3 donors, aged 43, 58, and 67 yr). In a previous study\textsuperscript{6} it was shown that orbital muscular tissues obtained from such a variety of donors, at surgery or postmortem, did not reveal apparent differences in the distribution of macrophages or T-cell subsets. Furthermore, if kept at 4°C, postmortem specimens do not show changes in form and structure or antigenicity for at least 12 hr\textsuperscript{13} (and personal communication (1980) Prof. Th. Vroom, pathologist) and can therefore be used in the same investigations as surgical specimens. Biopsy specimens of human skeletal muscle were obtained from the abdominal rectus muscle at cholecystectomy (4 donors, aged 30, 48, 54, and 57 yr) or at correction of ventral spondylitis (aged 57 yr), and from the femoral quadriceps muscle from a normal person (age 47 yr). The research followed the tenets of the Declaration of Helsinki, the nature of the investigation was explained fully to the patients (fresh tissue donors) and informed consent was obtained. None of the tissue donors had a known autoimmune disorder, or had recently been treated with corticosteroids.

After dissection the tissues were immediately frozen in isopentane cooled with liquid nitrogen and stored at -120°C.

**Immunohistochemistry**

Cryostat sections (8 µm) were cut with a Frigocut 2800 E cryostat (Reichert-Jung, Slough, UK) at -25°C and collected on gelatin-coated slides. On each slide 3–5 different tissue specimens (2–3 sections per specimen) were collected. Some of the intact muscles (exenteration specimens) were cross-sectioned at three sites: halfway, at one third and at two thirds of the muscles’ lengths. The sections were dried overnight at room temperature and fixed in acetone GR (Merck, Darmstadt, Germany) for 10 min. After air drying (30 min) slides were preincubated with phosphate buffered saline (PBS, pH 7.4) containing 0.3% H\textsubscript{2}O\textsubscript{2} and 0.1% NaN\textsubscript{3} for 10 min to inhibit endogenous peroxidase activity.\textsuperscript{14}

After washing (PBS) the sections were incubated (1 hr) with mouse monoclonal antibodies (see Table 1) diluted in PBS containing 1% bovine serum albumin (BSA) (Boseral, Organon Teknika, Nederland BV, Boxtel, The Netherlands). Then the sections were incubated for 1 hr with peroxidase conjugated rabbit anti-mouse immunoglobulin G serum (Dakopatts, Glostrup, Denmark) diluted in PBS containing 1% normal human AB serum (complement inactivated; Centraal Laboratorium Bloedtransfusiedienst, CLB, Amsterdam, The Netherlands). After 10 min incubation with 0.05% 3,3’-diaminobenzidine-tetra-HCl

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Specificity/Cluster Differential (CD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBM11</td>
<td>Monocytes, macrophages/CD12</td>
</tr>
<tr>
<td>DRC1</td>
<td>Follicular dendritic reticulum cells</td>
</tr>
<tr>
<td>DK22</td>
<td>HLA-DR, beta-chain (DR, DP, DQw1 not DQw5)</td>
</tr>
<tr>
<td>T3</td>
<td>T cells/CD3</td>
</tr>
<tr>
<td>T5</td>
<td>T cells/CD5</td>
</tr>
<tr>
<td>Ts</td>
<td>Suppressor/cytotoxic T cell subset/CD8</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cell subset/CD4</td>
</tr>
<tr>
<td>B</td>
<td>B cells/CD19, CD22, CD45R</td>
</tr>
<tr>
<td>TF-R</td>
<td>Transferrin Receptor on proliferating cells</td>
</tr>
<tr>
<td>PC</td>
<td>All proliferating cells, except G0 phase</td>
</tr>
<tr>
<td>IL-2-R</td>
<td>II-2 receptor of activated T cells/CD25</td>
</tr>
</tbody>
</table>

All monoclonal antibodies were obtained from Dakopatts (Glostrup, Denmark). Each batch of monoclonal antibodies was titrated and subsequently used at the optimal dilution in these investigations.
(Sigma) in PBS containing 0.01% H₂O₂ to detect peroxidase activity (in brown), they were weakly counterstained with diluted Mayer's hematoxylin (Merck), dehydrated and mounted in Entellan® (Merck). All incubations were carried out in a moist chamber at room temperature. Between incubations slides were washed with PBS (3 × 2 min). To obtain slides with high and constant quality, the staining procedures were carried out under standardized conditions, and, within an experiment all slides were processed simultaneously. Controls, necessary for the detection of nonspecific reactivity, included incubations with monoclonal antibodies against nonrelated proteins (anti-rat macrophage markers) and peroxidase conjugated antiserum (rabbit anti-fluorescein isothiocyanate). Sections of human lymph nodes were used as positive controls.

Microscopy
The slides were examined with a Leitz Orthoplan light microscope (Leitz Wetzlar GmbH, Wetzlar, Germany) at magnifications ×250, ×400, and ×1000. Profiles of immunopositive cells with a visible nucleus were quantified, using an ocular graticule at magnification ×400 (sample area about 0.1 mm²). Per cross-sectioned muscle, the cell number in 2 × 10 random frames was counted in the central area of two adjacent sections (total sample area about 2 mm²). The data were expressed as number of cells per square millimeter.

Image Analysis
The tissue distribution and morphology of immunopositive macrophages were analyzed in more detail with a semiautomatic image analysis system (VIDAS, Kontron), connected to a microscope (Zeiss, Axioskop, Fluotar ×25 objective) with a Sony XC-77 CE black and white charge coupled device (CCD) video camera (Sony Nederland BV, Badhoevedorp, The Netherlands). The histologic slides were transilluminated by a halogen light source with stabilized power supply. The light was filtered at the wavelength of maximum absorption of the diaminobenzidine reaction product (440 nm) to achieve maximal contrast between immunopositive macrophages and the surrounding tissue. The measuring algorithm was developed using the standard software and the built-in macro program generator of the VIDAS-system. The excellent contrast of the macrophages and the high and constant quality of the tissue sections enabled highly reproducible results with a fairly simple procedure. First the light intensity was calibrated using a double polarization filter attached to the microscope condenser and a real-time video look-up table; the brightest unstained areas of the real-time video image were adjusted to intensity value I = 200 (I_{max} = 256). Then a video image was averaged eight times (to suppress noise) and digitized as a matrix of 512 × 512 pixels, each pixel representing the local gray value (1 byte); the spatial resolution was 0.6 μm per pixel. Dark-current and shading corrections, to correct for local inhomogeneities in the sensitivity of the camera or for uneven illumination of the image, were followed by a single contour enhancement operation (Fig. 1A). Then a binary mask of the immunopositive objects (macrophages) was derived from the digitized video image on the basis of their (dark) gray values (Fig. 1B). A scrap procedure was performed to eliminate iso-

FIGURE 1. Semiautomatic image analysis of macrophages in extraocular muscle cross-sections. (A) Digitized video image of immunostained macrophages after dark-current and shading corrections and a contour enhancement operation. (B) Binary image of the macrophages resulting from automatic segmentation on the basis of (dark) gray values of the cells and removal of redundant structures (cell caps and processes) by a "scrap" procedure; on such binary images several parameters have been quantified simultaneously.
lated cellular processes from the mask, yielding about 95% nucleated cell profiles. Profiles of overlapping cells, which rarely occurred, were separated interactively (using a digitizer-tablet). Cell profiles touching the right or upper edge of the measuring frame were removed from the mask. After filling holes in the macrophage profile mask, the numeric density and the mean surface area of the macrophages were measured (cell profiles touching an edge of the measuring-frame were excluded from area or shape analysis). For each cross-sectioned extraocular muscle, 1 mm² was sampled (= 10 summed measurements) in the orbital and in the global muscle layer. In a separate series of measurements the shape distributions (dmax/dmin) of circa 600 macrophage profiles of lateral and inferior rectus muscle were compared to each other.

Statistics
Data were analyzed with a one-way analysis of variance followed by the Student-Newman Keuls test. The analysis was preceded by tests for homogeneity of variance (Bartlett's test; cf. Steel and Torrie15 and for the joint assessment of normality.16

RESULTS
General Observations
All orbital specimens showed good tissue preservation with no signs of ongoing pathologic processes. Moreover, negative immunostaining with either monoclonal antibodies to interleukin-2 receptor, the transferrin receptor, or to a cell proliferation marker confirmed that these tissues were devoid of any activated T cells and other proliferating cells. Control slides, where incubations with monoclonals were substituted by PBS/BSA, normal mouse serum or an irrelevant monoclonal antibody (DRC1), were negative. Muscle cells did not show immunopositivity with either anti-HLA-DR or any of the other monoclonal antibodies applied. Analysis of muscle fiber diameters and the percentage of area occupied by connective tissue in the cross-sections revealed that there are no significant differences in contractional state between the extraocular muscles. In the case of enucleation specimens, we selected the cases mentioned in the Materials and Methods section out of many more specimens handed to us, because in these sections the area occupied by connective tissue in relation to the area of muscle fibers in the cross-sections showed that we were looking at the belly of these muscles. In four of the eight cases it was possible to study the cell distribution in all four extraocular muscles, in the other cases only the lateral and medial extraocular muscles were available. The superior rectus not available in postmortem or enucleation specimens because the eyelids had to remain intact. Only in one case (a surgical exten-

teration for basal cell carcinoma) was it possible to study this muscle but general conclusions cannot be drawn from one specimen. For the surgical specimens it was not possible for obvious reasons to study extraocular and skeletal muscles obtained from the same donors nor was it possible for the postmortem specimens as the autopsy was limited to the exenteration procedure, but as the range in number of cells per square millimeter among different persons is within reasonable limits, this does not constitute a major concern.

T cells, macrophages, and HLA-DR positive cells
occurred dispersed in the epimysium, perimysium, and endomysium of both extraocular and skeletal muscles (Fig. 2), whereas B cells (CD19-, CD22- or CD45R-positive) were virtually absent. Some of these cells appeared to be closely associated with the vascular system (Figs 2B and 2C) and could be found perivascularly, in the vascular walls or intravascularly (lining up with the endothelium) in particular in small veins, but also in arterioles.

T cells (CD3-, CD5-, CD4- or CD8-positive cells) were round or oval and contained only little cytoplasm (Fig. 2A). CD4 however, also occurred on non-T cells: approximately one third of the CD4-positive cells were irregularly shaped and clearly do not represent T cells. In extraocular muscles T cells occurred solitary or in groups of 2–10 cells. In a cross-section about 0–4 of such groups are found, in skeletal muscle no such groups of T cells were present. Both macrophages and HLA-DR positive cells occurred in rounded, elongated or irregular dendritic shapes (Figs 2B, 2C and 2D) and have similar vascular relationships and tissue distribution patterns. HLA-DR was also expressed on the endothelium of some veins (Fig. 2C) and capillaries but not of arterial elements.

Quantitative Analysis of Cell Distribution

B cells (CD19-, CD22- or CD45R-positive cells) were virtually absent in both extraocular and skeletal muscle; in a few extraocular muscle sections a single B cell was sampled (Table 2).

T cells occurred either singularly or in groups. Because groups contain such variable cell numbers and were irregularly distributed, a T-cell group was counted as 1 cell. T cells appeared to be evenly distributed among the extraocular muscles, as their numbers did not vary much between the different types (Table 2). In the extraocular muscles most T cells were CD8-positive (suppressor/cytotoxic) and occurred two or three times more frequently than the CD4-positive T (helper) cells. In skeletal muscles, T cells occurred three times less frequently than in extraocular muscles and were predominantly of the helper subtype (CD4/CD8 ratio > 1). In all muscular tissues the number of cells positive with the pan-T cell marker CD3 almost equaled the sum of CD4 and CD8 positive T cells. Unexpectedly, the number of CD3-positive cells in extraocular muscles was up to seven times higher, and in skeletal muscle about four times higher, than with the pan-T-cell marker CD5. In lymph nodes the numbers of CD3 and CD5 positive T cells appeared to be similar.

Macrophages and HLA-DR positive cells occurred much more frequently in extraocular muscles than in skeletal muscles and showed an uneven distribution among the different types of extraocular muscles. The macrophage numbers in the inferior or medial recti muscles are almost twice those found in the lateral rectus or superior oblique muscles (P < 0.01). HLA-DR positive cells showed a similar skewed distribution, their numbers being highest in the inferior and medial rectus muscles, the differences with the lateral rectus or superior oblique muscles were however, smaller (P < 0.05). The ratio of HLA-DR positive cells to macrophages in this study, about 1:5 in all extraocular muscle types, was smaller than previously reported (1:3). To investigate this discrepancy we compared the HLA-DR immunopositivity in the postmortem tissue specimens and the fresh (surgical) tissue specimens. It appeared that the number of HLA-DR positive (nonendothelial) cells in autopsy extraocular muscles was about half of that observed in the surgical specimens (eg, for the rectus inferior 12.0 ± 5.5 and 27.2 ± 10.4 cells/mm² respectively, and for the rectus lateralis 9.0 ± 5.4 and 17.5 ± 9.3 cells/mm²). Apparently, the epitope recognized by the anti-HLA-DR monoclonal was relatively more susceptible to postmortem (protease) degradation. The autopsy extraocular muscle specimens did not differ from specimens

### Table 2. Distribution (Number/mm²) of Immunocompetent Cells in Extraocular Muscles and Skeletal Muscle

<table>
<thead>
<tr>
<th>Cell Type/CD Number</th>
<th>LR (n = 8)</th>
<th>IR (n = 8)</th>
<th>MR (n = 4)</th>
<th>SO (n = 4)</th>
<th>SM (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells/CD19, 22, 45R</td>
<td>≤1</td>
<td>≤1</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>T cells/CD3</td>
<td>14 ± 5</td>
<td>13 ± 8</td>
<td>11 ± 5</td>
<td>8 ± 4</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>T cells/CD5</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>T cells/CD4</td>
<td>3 ± 2</td>
<td>4 ± 2</td>
<td>4 ± 1</td>
<td>3 ± 2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>T cells/CD8</td>
<td>9 ± 3</td>
<td>8 ± 3</td>
<td>8 ± 1</td>
<td>5 ± 3</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>MΦ/CD68</td>
<td>63 ± 15</td>
<td>106 ± 26</td>
<td>105 ± 32</td>
<td>60 ± 19</td>
<td>13 ± 7</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>13 ± 8</td>
<td>19 ± 12</td>
<td>18 ± 10</td>
<td>9 ± 4</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

Data are presented as the total number of cells per mm² ± SD. LR, IR, and MR = lateral, inferior, and medial rectus muscle respectively; SO = superior oblique muscle; SM = skeletal muscle (5 × abdominal rectus muscle and 1 × femoral quadriceps). It was not possible to obtain all the muscles in all eight cases. MR and SO specimens were only available from the following donors: surgical exenteration for basal cell carcinoma postmortem (cardiac arrest and Alzheimer). One MR specimen was from an enucleation for intraocular melanoma and one SO specimen from a postmortem cervix cancer.
TABLE 3. Distribution (Number/mm²) of the Major Types of Immunocompetent Cell Subsets at One Third, Halfway, and Two Thirds Away From the Global Attachment Points of the Lateral and Inferior Recti Muscles

<table>
<thead>
<tr>
<th>Site of the Cross Section</th>
<th>One Third</th>
<th>Halfway</th>
<th>Two Thirds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral rectus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cell (CD4)</td>
<td>4 ± 2</td>
<td>5 ± 3</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>T cell (CD8)</td>
<td>8 ± 4</td>
<td>15 ± 2</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>Macrophage (CD68)</td>
<td>67 ± 19</td>
<td>60 ± 16</td>
<td>60 ± 16</td>
</tr>
<tr>
<td>Inferior rectus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cell (CD4)</td>
<td>4 ± 1</td>
<td>6 ± 4</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>T cell (CD8)</td>
<td>7 ± 4</td>
<td>12 ± 4</td>
<td>7 ± 6</td>
</tr>
<tr>
<td>Macrophage (CD68)</td>
<td>95 ± 23</td>
<td>93 ± 22</td>
<td>84 ± 14</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (n = 3).

obtained from surgery with respect to B cell (CD19-, CD22- or CD45R-positive), T cell (CD3-, CD5-, CD4- or CD8-positive) or macrophage (CD68) numbers.

Analysis of three inferior and lateral recti muscles (from three postmortem exenterations), cross-sectioned at one third, halfway, and at two thirds of the muscles’ lengths, revealed that CD4-positive T cells and macrophages are evenly distributed along the muscle length (Table 3). The number of CD8-positive T cells halfway both the inferior and lateral rectus muscles was however, approximately two times higher than at the other sites (no significant difference in inferior recti; in lateral recti: P < 0.05).

Semiautomatic Analysis of Macrophage Distribution

Semiautomatic image analysis confirmed the measurements performed with the ocular graticule, the inferior recti containing about twice as many macrophages as the lateral recti muscle (P < 0.01); this was the case for both orbital and global muscle layers. The mean size (area) of these macrophages did not vary significantly among the different muscle sites (Table 4). Shape analysis of about 600 macrophage profiles in the lateral and inferior recti muscles, showed that the shape distributions (dmax/dmin) in the two muscle types were similar (Fig. 3). These data implied that the observed differences in macrophage numbers among the muscle types do not relate to the size or shape, indicating that there were no site-dependent differences in activity state of these cells.

DISCUSSION

This is the first systematic and quantitative study concerning the distribution of immunocompetent cells in normal human extraocular muscles. It appeared that macrophages in particular, as defined with the highly specific pan-macrophage marker EBM11 (anti-CD68) but also HLA-DR positive macrophages did not seem to be caused by the predominance of a particular (morphologic) subset of these cells as their sizes and shape distributions in different muscles were similar. Furthermore, morphologic evaluation indicates that many of the HLA-DR positive cells are in fact macrophages that express HLA-DR. The proportion of this macrophage subset does not seem to vary much among the extraocular muscles as the ratio of HLA-DR positive cells to macrophages was similar in all extraocular muscle types. Previously, it was shown that many macrophages of the extraocular muscles express HLA-DR, and can be considered as potential antigen presenting cells. Antigen presenting cells are important immunoregulatory cells; they can induce primary immune responses, activate primed T cells and may even mediate self-tolerance. These cells may be very important for the local regulation of orbital immunity. Their particular distribution might relate to yet unknown intrinsic differences, such as immunogenicity, antigenicity or innervation, between normal extraocular muscles. The inferior and medial recti muscles for instance, which contain more macrophages, are innervated by the oculomotor nerve, whereas the lateral rectus and superior oblique muscles, containing less macrophages, are innervated by the abducens nerve and trochlear nerve respectively. Interestingly, in the extraocular muscles of rats similar cell distributions occur, which adds to the fundamental character of the observations.

Although the physiologic role for the heterogeneous distribution of macrophages and the presumed antigen presenting cells among normal extraocular muscles is still unclear, it might be of significance for

TABLE 4. Semiautomatic Analysis of Macrophage Distribution and Size in Orbital and Global Layers of Lateral (LR) and Inferior (IR) Recti Muscles

<table>
<thead>
<tr>
<th></th>
<th>Number/mm²</th>
<th>Area (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LR</td>
<td>IR</td>
</tr>
<tr>
<td>Orbital layer</td>
<td>48 ± 10</td>
<td>111 ± 35</td>
</tr>
<tr>
<td>Global layer</td>
<td>49 ± 15</td>
<td>90 ± 29</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD (n = 6).
the immunopathology of certain orbital immune disorders. In Graves’ ophthalmopathy, for instance, not all the muscles appear to be affected by the disease to the same extent. The superior group, the inferior and medial recti muscles are the most affected, whereas the lateral rectus and superior oblique muscles are far less affected.\textsuperscript{10,11,12,22} Interestingly, some of the most affected muscles contain the largest numbers of macrophages in normal persons. Furthermore, skeletal muscles, which appear unaffected by this autoimmune disease,\textsuperscript{9,24} contain relatively low numbers of these cells. The occurrence of Graves’ ophthalmopathy is strongly associated with Graves’ hyperthyroidism.\textsuperscript{9,11,24,25} In this respect, it might be of significance that macrophage-like cells in the extraocular muscles contain a high amount of cytoplasmic thyroid hormone receptors\textsuperscript{26} and that macrophages/monocytes may transform into antigen presenting cells after exposure to high concentrations of thyroid hormone.\textsuperscript{27} Antigen-presenting cells and macrophages have been claimed to play a role in the primary events of autoimmune diseases such as diabetes mellitus and Graves’ hyperthyroidism.\textsuperscript{28–31}

T cells may also be involved in the local regulation of immunity, which is indicated by the apparent sequestration of suppressor/cytotoxic T cells in the extraocular muscles. In extraocular muscles, the ratio of helper (CD4) to suppressor/cytotoxic T cells (CD8) is approximately 0.3–0.5, which is much lower than the ratio of around 1.0 found in the skeletal muscles or of approximately 2.0 as published for peripheral blood.\textsuperscript{32} Interestingly, in normal lateral and inferior recti muscles, the numbers of suppressor/cytotoxic T cells appears to be higher in the belly regions of the muscles than toward the extremities (Table 3).

The close anatomic relationships seen in extraocular muscle sections of macrophages, HLA-DR-positive cells or T cells with blood vessel endothelia, suggest involvement of the vascular system in the exchange of cells between the tissues and the blood. Relevant in this respect might be that the orbital muscle layer, which has a higher ratio of vascularization than the global layer,\textsuperscript{33} tends to contain larger numbers of macrophages. In addition, the expression of vascular adhesion molecules, activation or adhesion markers, such as HECA-452, HLA-DR, or CD36 (OKM5)\textsuperscript{6,7,8} may reflect the ability of the extraocular muscle endothelium to exert control over local cell recirculation. In this way, the endothelium may contribute to sequestration of suppressor/cytotoxic T cells or the differential distribution of macrophages in extraocular muscles.

**Key Words**

human, extraocular muscles, image analysis, immunocompetent cells, quantitative immunohistology

**Acknowledgment**

The authors thank Dr. P.I. Murray, ophthalmic surgeon, and Professor Dr. G. Asmussen, physiologist, for performing the autopsy exenterations; Drs. A.K.F. Tanka, J.G.M. Tinnemans, and F. van Coevorden, surgeons, for providing skeletal muscle specimens; and Dr. J. Bras, pathologist, for his advice and help with the autopsies.

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