Pathogenetic Mechanisms in Thyroid-Associated Ophthalmopathy

by

Anastasia Pappa

Institute of Ophthalmology
Moorfields Eye Hospital
Department of Clinical Ophthalmology
11-43 Bath Street
London, EC1V 9EL

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DEDICATION

To the memory of my mother, Helen Pappa,

and

to my teacher, Peter Fells.
ACKNOWLEDGEMENTS

I am deeply indebted to many individuals for making this work possible: to Professor Sue Lightman for training me as an independent scientist and for offering me the opportunity to work in her department; to Mr. Peter Fells for inspiring me to use my scientific skills - he became not only my teacher but friend and his home an oasis where there has always been a warm welcome; to Professor Tony Weetman for a stimulating collaboration, his ready availability and support when asked as well as his faith in me; to Dr. Colin Dayan for his provocative but always stimulating discussions and interest in this work as well as making himself available both to listen and inspire; to Dr. Petros Perros for critically reading parts of this manuscript; to Dr. Virginia Calder whose expertise with T cells saved me from the fate of many who have worked in this area; to Dr. Marian Ludgate for her brief but invaluable help with the antigenic preparations; to Dr. Charles Pennock for welcoming me into his laboratory and the daily, stimulating and unfailingly encouraging meetings with him; to Dr. Janet Stone for her knowledgeable support during the glycosaminoglycans study - Janet together with Charles have always made me feel at home while at Bristol pursuing the glycosaminoglycan analysis; to Dr. Peter Jackson for his great sense of humour, his warm hospitality at Cambridge during my numerous visits and stays for the assessment of glycosaminoglycans, and not least for willingly allowing the work to spread into the weekends and even his 1995 spring holiday; to Mr. John Lee who provided several of the non-TAO extraocular muscle specimens, to Mr. John Hungerford who supplied the extraocular muscle tissues for the antigenic preparation; to Professor Valerie Lund for providing the palatine tonsillectomy tissues; to Mr. John Webb for supplying the thyroid gland surgical
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I would also particularly like to thank Mr. George Comas for his continuous, unfailing, total support during all the years of my research work even from the time I was pursuing my Masters degree in medical immunology and for his encouragement to follow an academic career; Miss Jennifer Rignold for her invaluable personal advice at crucial times; Dr. John Leventides for his tutorials in statistics; and Dr. George Roussakis for his philosophical guidance and support during the writing of this thesis, and the associated papers.
"Ολβιος όστις της ιστορίας έσχε μάθησιν"

Happy is he who understands the inquiry

- *Euripides; 5 B.C*

"Πάσα τε επιστήμη χωρίζομένη δικαιοσύνης και της άλλης αρετής πανουργία, ου σοφία φαίνεται"

Every form of knowledge when sundered from justice and the rest of virtue is seen to be plain roguery rather than wisdom.

- *Plato; 5-4 B.C*
Thyroid-associated ophthalmopathy (TAO) is a disfiguring and potentially sight-threatening manifestation of autoimmune thyroid disease. Current therapy is limited to addressing the complications of the disease which include exposure keratitis, impairment of eye movements due to extraocular muscle (EOM) inflammatory involvement, and optic nerve compression with possible loss of vision. TAO is characterised by mononuclear cell infiltration, many of which are T cells, of the EOMs and/or the orbital fat/connective tissue with associated deposition of glycosaminoglycans (GAGs) in the interstitial spaces.

In this thesis, the presence and distribution of the vascular adhesion molecules ICAM-1, ELAM-1, VCAM-1 and the leukocyte integrins CD11a/CD18, CD11b/CD18, CD11c/CD18 were investigated, by immunohistochemistry, on EOM biopsies harvested from early, active and late, inactive TAO patients as well as non-TAO strabismus control subjects. Because of small biopsy size, on different EOM biopsies collected from the same groups of patients the mononuclear cell infiltrate as well as the expression and localisation of HLA-DR were also characterised immunohistochemically. Further EOM biopsies were taken from TAO patients and the infiltrating T cells were isolated and expanded in vitro with mitogen. Their phenotype was determined by FACS analysis and compared to peripheral blood-derived T cell lines, grown in vitro in the same way, from the same patient. Cytokines present in the supernatant after mitogen stimulation of the T cell lines, were assayed by ELISA techniques. Moreover, the pattern of cytokine gene expression in EOMs was studied ex vivo in biopsies taken from different TAO patients, and results were compared with the data derived from the T cell lines. With the T cell lines from two patients, proliferation assays were carried out using antigens derived from thyroid gland, EOM and a TSH-receptor preparation. In addition, the presence and localisation of GAGs on TAO and control EOM biopsies was examined by transmission electron microscopy and immunogold staining. Serum hyaluronan was measured using a radioimmunoassay in patients with TAO as well as control subjects, and urinary GAG levels assessed by photometric quantitation of hexuronic acid after reaction with carbazole. Finally, the excretion pattern of the urinary GAGs was determined by means of discontinuous electrophoresis.
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Anastasia Pappa  
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<th>Abbreviation</th>
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<tr>
<td>μm</td>
<td>micro-metre</td>
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<tr>
<td>ACHE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>AEC</td>
<td>amino-ethyl-carbazole</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APES</td>
<td>3-amino propyl tri-ethoxy silane</td>
</tr>
<tr>
<td>ATD</td>
<td>autoimmune thyroid disease</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD11a/CD18</td>
<td>(or LFA-1)</td>
</tr>
<tr>
<td>CD11b/CD18</td>
<td>(or Mac-1)</td>
</tr>
<tr>
<td>CD11c/CD18</td>
<td>(or p150,95)</td>
</tr>
<tr>
<td>CD4^</td>
<td>helper/inducer T cells</td>
</tr>
<tr>
<td>CD44</td>
<td>hyaluronic acid receptor</td>
</tr>
<tr>
<td>CD8^</td>
<td>suppressor/cytotoxic T cells</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cGy</td>
<td>centiGrays</td>
</tr>
<tr>
<td>CPC</td>
<td>cetyl pyridinium chloride</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulphate</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DS</td>
<td>dermatan sulphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribo-nucleic acid</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>ECD</td>
<td>extracellular domain</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ELAM-1</td>
<td>endothelial leucocyte adhesion molecule-1; (E-selectin)</td>
</tr>
<tr>
<td>EOM</td>
<td>extraocular muscle</td>
</tr>
<tr>
<td>FACS</td>
<td>flow cytometric analysis</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
</tr>
<tr>
<td>GD</td>
<td>Graves’ disease</td>
</tr>
<tr>
<td>GH</td>
<td>(pituitary) growth hormone</td>
</tr>
<tr>
<td>GlyCAM-1</td>
<td>glycosylation-dependent cell adhesion molecule-1</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan, hyaluronic acid</td>
</tr>
<tr>
<td>HABPs</td>
<td>hyaluronan binding proteins</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venules</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>hrIL-2</td>
<td>human recombinant IL-2</td>
</tr>
<tr>
<td>HS</td>
<td>human serum</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulphate</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HT</td>
<td>Hashimoto's thyroiditis; Hashimoto’s disease</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1; (CD54)</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>INCAM-110</td>
<td>Endothelial VCAM-1</td>
</tr>
<tr>
<td>IOP</td>
<td>intraocular pressure</td>
</tr>
<tr>
<td>KS</td>
<td>keratan sulphate</td>
</tr>
<tr>
<td>LAK cells</td>
<td>lymphokine activated killer cells</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function associated antigen-1</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Mφs</td>
<td>macrophages</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Mac-1</td>
<td>macrophage antigen-1</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MoAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>Nk cells</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>nm</td>
<td>nano-metre</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor type 1</td>
</tr>
<tr>
<td>PB</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>peridinin chlorophyll protein</td>
</tr>
<tr>
<td>pg/ml</td>
<td>picogram/milli-litre</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>PHA-L</td>
<td>phytohaemagglutinin-L; leucoagglutinin</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>PLT</td>
<td>progressive lowering of temperature</td>
</tr>
<tr>
<td>PM</td>
<td>primary myxoedema</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SI</td>
<td>stimulation index</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>soluble ICAM-1</td>
</tr>
<tr>
<td>sIL-1R</td>
<td>soluble IL-1 receptor</td>
</tr>
<tr>
<td>sLe^a</td>
<td>sialyl Lewis x</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SS-RT</td>
<td>SuperScript II reverse transcriptase</td>
</tr>
<tr>
<td>TAO</td>
<td>thyroid-associated ophthalmopathy</td>
</tr>
<tr>
<td>TCR V-r</td>
<td>TCR variable region</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TECs</td>
<td>Thyroid epithelial cells</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>RFCs</td>
<td>thyroid follicular cells</td>
</tr>
<tr>
<td>Tg</td>
<td>thyroglobulin</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TPO</td>
<td>thyroid peroxidase</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>TSH-R Ab</td>
<td>TSH-R antibody</td>
</tr>
<tr>
<td>TSH-R</td>
<td>TSH receptor</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1, E-selectin</td>
</tr>
<tr>
<td>VLA</td>
<td>very late activation</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
</tbody>
</table>
Chapter 1

INTRODUCTION
1.1 OVERVIEW

Thyroid-associated ophthalmopathy (TAO) is a disfiguring and potentially sight-threatening disease, for which treatment is still inadequate compared with the treatment for hyper- or hypo-thyroidism. There is therefore a need for more effective and, possibly, more specific forms of therapy. For such therapies to be developed, a better and more sophisticated understanding of the pathogenetic mechanisms of TAO is required.

The experiments described in this thesis have examined three different elements in the pathogenesis of TAO which may offer opportunities for improved monitoring and/or treatment of this disease. Firstly, the extraocular muscle (EOM) mononuclear cell infiltrate as well as human leukocyte antigen (HLA)-DR and adhesion molecule expression and localisation in relation to disease activity has been studied. Secondly, the phenotype, cytokine profile, and antigenic reactivity of the EOM infiltrate has been examined. Finally, the localisation, composition, and excretion of glycosaminoglycans responsible for the EOM enlargement have been characterised.

To place these studies in context, what is currently known of the pathogenesis of TAO and its relationship to autoimmune thyroid disease will initially be reviewed. Background information relating to adhesion molecules, T cell cytokines and glycosaminoglycans has also been included.

1.2 IMMUNOLOGY OF AUTOIMMUNITY

1.2.1 Classification: Organ-specific versus systemic autoimmune disease

Autoimmune diseases are characterised by the spontaneous development of
immune responses to self antigens resulting either in tissue damage or the generation of autoantibody responses which interfere with normal physiology\textsuperscript{[1]}. Traditionally, they are divided into systemic autoimmune disease, e.g. rheumatoid arthritis, systemic lupus erythematosus and related collagen vascular diseases, as well as organ-specific autoimmune diseases. Examples of the latter are shown in Table 1.1.

**Table 1.1 Organ-specific autoimmune diseases**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Auto-Antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 diabetes mellitus</td>
<td>Glutamic acid decarboxylase, Insulinoma-associated protein 2 (IA-2), insulin, proinsulin, phogrin</td>
</tr>
<tr>
<td>Graves' disease</td>
<td>Thyroglobulin, TPO, TSH-receptor</td>
</tr>
<tr>
<td>Hashimoto's disease</td>
<td>Thyroglobulin, TPO, TSH-receptor, sodium-iodide symporter</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Myelin basic protein, Proteolipid protein</td>
</tr>
<tr>
<td>Myasthenia Gravis</td>
<td>Acetylcholine receptor $\alpha$-subunit</td>
</tr>
<tr>
<td>Addison's disease</td>
<td>21-hydroxylase enzyme</td>
</tr>
<tr>
<td>Primary Biliary Cirrhosis</td>
<td>Pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>Uveitis</td>
<td>Retinal soluble antigen (S-Ag)</td>
</tr>
</tbody>
</table>

In systemic autoimmune diseases, circulating autoantibodies typically recognise ubiquitous self-proteins such as DNA, histones and riboproteins. However, not all tissues are targetted by the autoimmune reaction and inflammation is typically focussed on the joints, the renal glomerulus and the blood vessels in a non-antigen specific manner.

In contrast, in organ-specific autoimmune diseases, the immune response remains restricted to antigens expressed in the target tissue. In the last decade, many of the autoantigens recognised in individual diseases have been identified (Table 1.1). Note that the autoimmune response although, tissue specific, has generally not proved to be protein specific. Typically several self-proteins and several epitopes on each self-protein are recognised in a single individual \textsuperscript{[2,3]}. 
Interestingly, the same or a related autoantigen may be expressed in a different self-tissue which escapes autoimmunity. For example, glutamic acid decarboxylase and insulinoma-associated protein 2 (IA-2), both target antigens in Type 1 diabetes mellitus are also expressed in the brain and neuroendocrine tissues respectively and yet the autoimmune response remains restricted to the islet β-cells.

1.2.2. Role of T cells in organ-specific autoimmunity

Although autoantibodies are most easily studied, work in animal models strongly suggests that T cells play a central role in the pathogenic process. Transfer of CD4\(^+\) T cell clones specific for target tissue-derived autoantigens have been shown to transfer disease in both induced models, such as experimental allergic encephalomyelitis and in spontaneous models such as the non-obese diabetic (NOD) mouse model of diabetes. However, in intact animals, CD8\(^+\) cells also appear to play a role. For example, in NOD mice treatment with anti-CD8 antibodies from two to five weeks of age protects animals from developing both insulitis and diabetes suggesting that CD8 cells play a role in early islet cell pathology. This conclusion is supported by the recent observation that disease can be transferred with CD8\(^+\) T cell clones from young mice.

1.2.3. Mechanisms of initiating T cell activation

The mechanisms responsible for initiating the activation of self-reactive T cells in human spontaneous autoimmune disease remain unclear. Mechanisms proposed include molecular mimicry of self antigen by pathogens, stimulation of T cells by bacterial or viral superantigens and spontaneous presentation of antigen by the target tissue itself. Evidence in favour of each mechanism remains incomplete.
In molecular mimicry, cross-reactivity between a foreign antigen from a micro-organism and a self-antigenic epitope is proposed to result in activation of autoreactive T cells \[^9\]. In animal models, organ-specific autoimmune disease has been induced by immunisation with peptides derived from microbial antigens \[^10,11\] but only in the case of Theiler’s murine encephalomyelitis virus induced demyelinating disease has infection with a live microorganism been clearly shown to result in autoimmunity \[^12,13\]. In humans, cross-reactivity between autoantigen reactive T cell clones isolated from humans and microbial antigens has now been demonstrated in multiple sclerosis \[^14,15\], diabetes \[^16\] and primary biliary cirrhosis \[^17\]. In addition, it has become increasingly recognised that cross-reactivity can occur between antigenic peptides that bear little relationship in primary structure, particularly when the interactions are of low affinity \[^18\] , increasing the possibilities for molecular mimicry.

Superantigens are proteins produced by viruses or bacteria that are capable of activating large numbers of T cells by binding to a subset of \( \text{V}\beta \) segments in the T cell receptor \( \beta \)-chain. As a result, these proteins are attractive candidates for a mechanism of “accidental” activation of self-reactive T cells by pathogens and their ability to induce disease has been shown in EAE \[^19\]. In 1994, Conrad \textit{et al.} noted an expansion of \( \text{V}\beta\)-7 positive T cells in the pancreas of two patients with insulin-dependent diabetes and reported that such a T cell expansion could be reproduced by membranes from inflamed but not healthy islets \[^20\]. Recently, the same group has isolated a novel retrovirus able to selectively expand \( \text{V}\)-beta 7 cells and found it to be present in the blood of ten patients with diabetes and the islet cell supernatants from affected individuals but not controls \[^21\]. These findings await further confirmation.
and evidence that expression of the retroviral superantigen is a cause rather than a result of islet inflammation.

A role for spontaneous presentation of antigen by target tissues in triggering T cell activation has been proposed in the maintenance phase of autoimmune disease \[22\]. However, in transgenic mouse models in which a viral protein (lymphocyte choriomeningitis virus) is expressed on islet cells under the control of the insulin promoter disease does not develop unless the animals are separately infected with live virus \[23\], suggesting that endogenous presentation is unlikely to be responsible for initiating T cell activation.

It is important to note that the different possible mechanisms of T cell activation described above are not mutually exclusive. The trigger may differ in different individuals but yet result in pathologically similar autoimmune disease due to spreading of the T cell response to different autoantigens from the same tissue. This process of epitope spreading has been clearly shown to occur in animal models and is therefore discussed in further detail below.

**1.2.4. Epitope spreading and the T cell response in organ-specific autoimmune disease**

If molecular mimicry is the trigger for T cell activation in autoimmunity, this mechanism would not be expected to directly activate T cells recognising multiple different epitopes on distinct self-antigens. A process of diversification of the immune response after initial activation is required. Direct evidence for such a process, referred to as “epitope spreading” \[24\] has now been obtained in both induced \[25\] and spontaneous animal models (NOD mouse) \[26\] of organ-specific autoimmunity. These findings may therefore explain the diversity of autoantigen reactivity seen in human disease.
The exact mechanism by which this important process occurs, remains unclear. Many authors consider that tissue destruction as a result of autoimmunity to one self antigen simply leads to the release of further self-antigens for presentation by professional antigen presenting cells\textsuperscript{[13]}. However, this seems unlikely to be the complete explanation as tissue trauma or viral inflammation does not routinely lead to autoimmunity. Evidence for a role for non-professional presentation of antigens by target cells has been provided in thyroid autoimmunity\textsuperscript{[3,27]}. Such a mechanism might mediate epitope spreading. This will be discussed further in consideration of the immunology of thyroid autoimmunity itself in section 1.3.2.

### 1.2.5. T cell regulatory mechanisms and autoimmune disease

High affinity self-reactive T cells are likely to be deleted during thymic development\textsuperscript{[28]}. However, when the self-peptide-MHC complex is present at low concentration in the thymus and/or the T cell receptor has low affinity for this complex, positive selection may result, enabling such cells to pass into the peripheral lymphoid system. T cells reactive to self antigens can certainly be isolated from normal individuals\textsuperscript{[2]} and presumably have the potential to cause autoimmune disease if activated by the mechanisms described in sections 1.2.3 and 1.2.4. There is therefore a clear requirement for regulatory mechanisms to prevent this occurring and maintain so-called “peripheral tolerance”. Recent studies have identified an increasing number of such mechanisms, some or all of which may be defective in autoimmune disease.

**5a) Role of co-stimulatory molecules**

In the absence of co-stimulatory molecules (such as B7-1, B7-2 and CD40) providing a “second signal”, presentation of antigen to T cells results in anergy or
apoptosis rather than activation\textsuperscript{[29]}. Hence if self-antigens were presented in this way by appropriate antigen presenting cells, the result would be peripheral tolerance rather than autoimmunity. However, this is a difficult hypothesis to test directly \textit{in vivo}. Many groups have therefore studied the effects of blocking co-stimulatory molecules or their T cell ligands (e.g. CD28, CTLA-4) in models of autoimmune disease. Interestingly, the results have been conflicting. Early treatment with the blocking fusion protein, CTLA-4-Ig, prevents EAE\textsuperscript{[30]} but treatment during disease onset exacerbates disease\textsuperscript{[31]}. In contrast, NOD mice in whom the CD28 gene has been deleted, or who are transgenic for CTLA-4-Ig expression, develop a more aggressive form of autoimmune diabetes\textsuperscript{[32]}. The timing of exposure to different co-stimulatory stimuli therefore appears important in maintaining self-tolerance.

\textbf{(5b) Regulation by antigen presenting cells}

Antigen presenting cells provide additional stimuli to T cells beyond T cell receptor engagement and co-stimulation. The cytokine environment in which antigen presentation occurs may alter the result from autoimmunity to peripheral tolerance. Transgenic expression of IL-4 or TNF-\(\alpha\) by islet cells in the NOD mouse has been shown to prevent the development of diabetes\textsuperscript{[33,34]}. The protective effects of TNF-\(\alpha\) suggests that these effects are not simply due to a bias of the T cell repertoire towards T helper 2 (Th2) cells. Interestingly, NOD-IL-4 transgenic mice are protected from diabetes after adoptive transfer of splenocytes from diabetic donors but NOD-TNF mice are not.

Evidence of deletion of self-reactive CD8 cells by lymphoid antigen presenting cells has recently been presented by Kurts \textit{et al.}\textsuperscript{[35]}. In this model, CD8 cells carrying a T cell receptor specific for ovalbumin (OVA) were injected into transgenic animals expressing OVA on islet \(\beta\)-cells. The CD8\(^{+}\) cells were activated,
underwent initial proliferation, infiltrated the islets to some extent and subsequently underwent deletion. Presentation of OVA appeared to be occurring via bone-marrow derived cells, not by the islet cells themselves, implying transfer of antigen from the islet cells. The exact mechanisms involved remain uncertain but this may represent an important homeostatic mechanism, at least with respect to CD8 cells.

(5c) Role of regulatory T cell subsets

A role for active regulation of T cell responses by other T cells has long been proposed and demonstrated in animal models $[36-38]$. The exact T cell subset and mechanism involved remains the subject of debate, but evidence in favour of a role for $\gamma\delta$ T cells, particularly after mucosal tolerance induction, has been provided $[39,40]$. Attention has also focussed on CD4 $\alpha/\beta$ T cells which secrete TGF-\(\beta\), sometimes referred to as Th3 cells $[41]$ and on cells that additionally produce high levels of IL-10 $[42]$. However, attention has also been directed to a rare CD4- CD8- T cell subset with restricted T cell receptor usage, and expression of the surface antigen NK1.1 $[43]$. These latter T cells appear to be expanded in individuals who fail to progress to diabetes. NK1.1 cells express IL-4 in addition to IFN-\(\gamma\) $[44]$ but direct evidence for their regulatory role in humans has yet to be provided.

(5d) Role of Fas/Fas-Ligand

Molecules of the TNF/TNF receptor superfamily have been shown to be present on T cells and to be able to mediate apoptosis of both target cells and T cells themselves. CD95L (Fas Ligand) delivers an apoptotic signal on binding to CD95 (Fas/APO-1). Deletion of potentially self-reactive T cells expressing CD95 by this mechanism may therefore represent a means of protection against autoimmunity. Defects in this system as seen in lpr and gld mice result in a lupus-like disease and a comparable condition termed "autoimmune lymphoproliferative syndrome" has been
identified in humans \[45\].

The recent observation that CD95L is expressed by thyroid epithelial cells \[46\] raises the possibility of a role for this molecule in protecting thyroid cells from attack by T cells in autoimmune thyroiditis \[47\]. However, paradoxically, transgenic expression of CD95 ligand on islet cells in NOD mice results in accelerated autoimmunity, emphasising the complexity of this system \[48,49\]. The recent identification of additional TNF family members that also carry "death domains" such as DR4 and DR5 and a non-functioning homologue which may act as a "decoy receptor" homologue, TRID/DcR1, may begin to clarify some of these complexities \[50\].

**1.2.6. The Th1-Th2 paradigm in autoimmunity**

The identification in 1986 of subsets of CD4 cells distinguishable by their cytokine production \[51\], lead to studies of cytokine production by T helper cells involved in autoimmunity. CD4\(^+\) T cell clones able to transfer disease in EAE \[52\] and NOD mice \[53\] were found to be of the Th1 subset (IFN-\(\gamma\) producing). This lead to the paradigm that pathogenic autoimmune responses result from a preponderance of Th1 cells over cells which produce IL-4, IL-5 and IL-10 (Th2) \[54\]. Although the reverse is the case in some autoimmune models such as HgCL2 induced glomerulonephritis, this paradigm appeared to hold true in EAE following the demonstration that IL-10 levels were increased in the central nervous system in animals in remission \[55\] and that adoptive transfer of Th2 cells specific for autoantigen could protect mice from developing disease.

However, further studies appear to indicate that the circumstances under which T cells differentiate into either a Th1 or Th2 phenotype can alter their
pathogenic potential. For example in EAE, Th2 lines derived after treatment with anti-B7-1 antibodies to induce immune deviation were able to transfer protection from disease whereas Th2 lines generated by other means did not. Furthermore, administration of IL-4\(^{[38]}\) or IL-10\(^{[56]}\) after disease induction was found to exacerbate disease. In NOD mice, transfer of antigen-specific Th2 cells appears not to protect against diabetes\(^{[57]}\) but deviation of cells to the Th2 phenotype by administration of an IL-12 antagonist from the age of three weeks is successful in preventing disease\(^{[58]}\). To add to this complexity, Th2 cells both in EAE and NOD mice have been shown to mediate aggressive disease in hybrid immunocompromised (SCID) mice\(^{[53,59]}\), suggesting that an additional regulatory immune cell is required to mediate protection against disease.

### 1.2.7. An overview of immunological mechanisms in autoimmunity

A current working hypothesis of the pathogenesis of organ-specific autoimmune disease would suggest that low affinity, self-reactive T cells escape negative selection in the thymus and are present in the peripheral blood of most individuals. However, for autoimmune disease to occur, these cells must become “accidentally” activated by molecular mimicry or superantigens. Tissue damage due to the initiation of an autoimmune response then leads to presentation of other self-antigens within the target tissue and “epitope spreading” in the autoimmune response to a range of proteins and peptide epitopes present in the target tissue.

A further requirement for the generation of autoimmune disease is the failure of regulatory mechanisms including regulatory T cell subsets, apoptosis induction by Fas ligand or anergy induction by the absence of co-stimulatory signals on target cells. The result is typically the generation of CD4\(^+\) T cells with a preponderance of
the Th1 phenotype that are able to mediate tissue damage.

1.3 AUTOIMMUNE THYROID DISEASE

Autoimmune thyroid diseases (ATD) are common, affecting up to 1% of the population [60]. Both ends of the spectrum of ATD, overactivity and underactivity, are consequences of thyroid autoimmunity. Thyroid autoimmunity shares many features with other autoimmune endocrinopathies, such as chronic active hepatitis, primary biliary cirrhosis, myasthenia gravis or insulin-dependent diabetes mellitus (IDDM), and is regarded as the "prototype" of organ-specific autoimmune endocrine disease as it was the first to be described [61].

1.3.1 GRAVES' DISEASE (AUTOIMMUNE HYPERTHYROIDISM)

1.3.1.1 Clinical features of Graves' disease

Hyperthyroidism is a common disease affecting, according to the Whickham survey of 1977, 1.9% of women and 0.16% of men [60]. Around 80% of hyperthyroid patients have Graves’ disease, characterised by a diffuse goitre, thyrotoxicosis and ocular changes (TAO). It can occur at any age, but most commonly occurs between the age of 20-45 years [62]. Using sensitive diagnostic techniques, the presence of TAO can be demonstrated in the majority of patients with Graves’ disease [63-65]. A smaller percentage of patients with Graves’ disease reveal the combined presence of TAO and pretibial dermopathy (myxoedema), and even more rarely, finger or toe acropachy [63].

1.3.1.2 Natural history of Graves’ disease

The natural course of Graves’ disease has not been studied extensively as
institution of treatment usually follows diagnosis nowadays\cite{66}. Early observations in 1908 indicated that 25% proved fatal, 25% became chronic, and 50% became more or less cured\cite{67}. The prognosis was better in children than in adults, and better in private patients who could rest than in the poor\cite{68}. Also, Hale White reported in 1910 that between 30% and 48% of untreated patients remained “quite well”\cite{68}. On the basis of this kind of clinical evidence a spontaneous remission rate of 30% became generally accepted\cite{69}. More recently, based on patients treated with β-blockers only, a spontaneous remission rate of closer to 10% has been reported. In addition, 20-30% of patients convert to hypothyroidism as long as 20 years after antithyroid drug treatment\cite{70} or as a late development (10-20 years) after thyroid surgery (10%-18%)\cite{71}. Overall, the limited data available would suggest that in the absence of treatment Graves’ disease may remit spontaneously\cite{72}, be followed by permanent hypothyroidism\cite{73,74}, persist\cite{75}, or swing between phases of hyper- and hypothyroidism.

1.3.1.3 Thyroid histopathology in Graves’ disease

Glandular hyperplasia is the most common histologic manifestation of Graves’ disease\cite{76}. The term “diffuse toxic goitre”, designated for the thyroid gland of Graves’ disease, denotes that the gland is both enlarged and uniformly affected. Diffuse toxic goitres vary in consistency from softer than normal to firm and rubbery. The cut surface is red and glistening\cite{77}. Microscopically, the follicles are small, lined by hyperplastic columnar epithelium (unless presurgical iodide treatment has been administered), and contain scant colloid that displays much marginal scalloping and vacuolisation. Lymphocytic infiltration (60-70%)\cite{78}, sometimes associated with germinal centre formation, is often seen in patients with classic
hyperthyroid Graves' disease \[79\], although this lymphocytic infiltration is not as heavy as in Hashimoto's thyroiditis. Usually, the lymphoid cells are distributed in the interfollicular stroma and do not encroach on the follicles themselves. Surprisingly, plasma cells are rare \[78\] in Graves' disease. Vascularity is increased. Fibrosis is unusual as is oxyphilia (follicular cells with pink granular cytoplasm) unless the Graves’ disease is long-standing \[80\].

### 1.3.1.4 Genetic and environmental factors in the aetiology of Graves’ disease

There is certainly a genetic predisposition \[81-88\]. Graves’ disease runs in families \[89,90\] and the occurrence in relatives correlates with the presence of TSH-R antibodies \[91\]. Although considerable information has been accumulated on HLA associations in Graves’ disease, the results and their implications have not been as clear as those for type 1 diabetes mellitus \[92\]. The relative risk associated with HLA-DR3/DQw2 gene within Caucasians has varied widely from figures as high as 7.4 \[81,93\] to an inability to demonstrate any association \[94\]. The extension of studies into the DP and DQ regions of class II, using PCR and allele-specific oligonucleotide probes, has suggested no association with the genes of the DQB and DPB alleles, as observed by Weetman et al. \[95\]. In contrast, an increase in the frequency of the specificity DQA1*0501 in Graves’ patients as compared with controls, giving a relative risk of 3.7, was shown in a Caucasian population \[82\]. This association still remained significant (with a relative risk of 3.4) after exclusion of the HLA-DR-positive subjects. In the context of disease severity, as assessed by relapse after antithyroid drug therapy, data from McGregor’s group suggested associations between certain class II alleles (DQA2U) and disease relapse \[83\]. The same group, using combined segregation and linkage analysis, studied 217 members of 21
families of patients with Graves' disease and demonstrated cosegregation of HLA-DR17 (the former DR3) with Graves' disease within the families studied \cite{84,85}. The authors, however, supported the view that HLA-DR17 is not the only disease susceptibility allele involved.

Also, a number of studies have examined associations between Graves' disease and immunoglobulin allotypes. Within both Caucasian \cite{86,87} and Japanese \cite{88} populations, there is a failure for studies to agree and conclusions remain uncertain. Genetic associations of Graves' patients with and without TAO were studied in two patient populations in North East England \cite{96}. Phenotype and gene frequencies were compared with a control unaffected population of the region \cite{96}. The authors concluded that a number of genes (HLA-B8 and DR3) rather than a single gene are involved in susceptibility to Graves' disease, with or without TAO. However, patients with TAO were found to have an increased frequency of P blood group \cite{96}. An association between a specific restriction fragment length polymorphism (RFLP) of the hsp-70 gene and Graves' disease has also been reported \cite{97}.

Environmental factors thought to be involved include: (a) increased ingestion of dietary iodine in situations of either previously normal or low iodine intake \cite{98-102}, (b) infectious agents \cite{103,104} (viruses, bacteria), (c) stress \cite{105-113}. Yersinia enterocolitica infections \cite{114-118} as well as retroviruses \cite{119} have been implicated in causing Graves' disease. However, the significance of these findings remains uncertain \cite{120-122}. The role of stressful events in the onset of Graves' disease still remains controversial. Although stress may be a precipitant for the onset of disease in those who are predisposed to autoimmune thyroid disorders, it is not of its own sufficient to cause disease. Presumably, other factors involved in disease susceptibility need to be present prior to, or concomitantly with, exposure to stress.
Overall, Graves' disease best fits a polygenic, multifactorial model of disease in which (a) genetically susceptible individuals are (b) exposed to a constitutional or environmental insult as a result of which (c) immune system activation triggers the B-cell production of TSH-R antibodies which leads directly to (d) the induction of hyperthyroidism \cite{95,123}.

### 1.3.2 IMMUNOLOGY OF GRAVES' DISEASE

#### 1.3.2.1 Role of anti-TSH receptor antibodies in Graves' disease

In 1956, a "long-acting thyroid stimulator" was found in the sera of patients with Graves' disease \cite{124,125} and by 1964 it has been established that this non-physiological thyroid stimulator was in fact an immunoglobulin (Ig) \cite{126}. Indeed in the same year it became clear that the transient hyperthyroidism that may occur in neonates born to mothers with Graves' disease was caused by the transplacental passage of these IgG thyroid-stimulating molecules \cite{127}. However, it was not until 1989 that the TSH receptor itself was finally cloned \cite{128}.

It is now generally accepted that Graves' disease is due to agonist antibodies to the TSH-R \cite{95,123,129}. By binding to the receptor, these stimulate thyroid function, which becomes manifest clinically by the signs and symptoms of thyrotoxicosis. Debate over the central role of anti-TSH receptor antibodies in causing the thyrotoxicosis has continued to be fuelled by the inability to detect such antibodies in all patients who clinically have Graves' disease. However, developments in assay systems for TSH-R antibodies now mean that this activity is detectable in at least 95% of all patients with untreated hyperthyroid Graves' disease \cite{95} suggesting that this lack of correlation is due to technical limitations \cite{130}.
Chapter 1: Introduction

Note that not all anti-TSH receptor antibodies have agonist activity. Indeed, although a large number of anti-TSH receptor antibodies monoclonal antibodies have been generated in vitro, none of these have been shown to have agonist activity. Antibodies able to compete with TSH for receptor binding are present in up to 24% of patients with Hashimoto’s thyroiditis and in at least 20% of cases they seem to block the action of TSH in vitro, since their spontaneous disappearance results in recovery of hypothyroidism \(^{[131]}\). It is likely that the crystal structure of the TSH receptor will require to be solved along with the exact binding site of anti-TSH receptor antibodies from Graves’ disease before the basis of the agonist activity is understood.

### 1.3.2.2 Auto-antigens recognised by T cells in Graves’ disease

Auto-antibodies directed at thyroglobulin (Tg) and thyroid peroxidase (TPO) in addition to the TSH-R are common in Graves’ disease indicating that the autoimmune response is not limited to the TSH-receptor. Studies of the antigens recognised by T cells in Graves’ disease is technically more difficult, but antigen specific T cells have been cloned from diseased thyroid tissue in one individual and some of these T cells responded to Tg (3%), or TPO (approximately 40%) as well as thyroid epithelium \(^{[3,27]}\). Additional clones have been found in the latter study to be unresponsive to TPO or Tg, but proliferated vigorously when exposed to autologous thyroid epithelium. More recently, the same group reported the identification of TSH-R specific T-cell clones from Graves’ disease thyroid tissue \(^{[132]}\) using autoantigen-transfected EBV-transformed B-cell lines \(^{[133]}\). Using a spectrum of peptides derived from the sequence of the extracellular domain of the TSH-R, and examining peripheral blood T cell proliferation in response to these peptides Tandon
et al. have suggested that the epitopes recognised by Graves’ disease PBMCs are heterogeneous, between and within individual patients. Taken together, these studies suggest that the T cell response in Graves’ disease is heterogeneous but highly focused on thyroid specific proteins.

Quaratino et al. have studied antigen recognition by TPO-reactive T cell clones derived from Graves’ thyroid tissue in great detail. The pattern of antigen recognition and the use of TCR molecules they observed was remarkable. Of three distinct clones recognising TPO residues 535-551, one expressed two in frame TCR Vα chains and two shared identical Vβ chains but differed only in the J region of the Vα chain. Furthermore, the clones were found to recognise two distinct but highly overlapping epitopes within the same amino-acid sequence, despite the fact that all clones were restricted by the same HLA-DQ element. Molecular modelling indicated that despite much sequence homology, these epitopes bound to DQ in different conformations and using separate anchor residues.

1.3.2.3 T cell receptor (TCR) variable (V) region usage in Graves’ disease

An overall restriction of T cell receptor (TCR) usage in some models of autoimmune disease has stimulated interest for similar studies, in thyroid disease also. Using reverse transcription-polymerase chain reaction (RT-PCR), Davies et al. provided evidence of marked restriction in T-cell receptor Vα chain usage in intrathyroidal infiltrates from Graves’ patients. When such studies were extended in the severe combined immunodeficiency (scid) mouse animal model using “organoids” obtained by transferring thyroid and lymphoid cells from patients with Graves’ disease, the original restricted usage of the Vα families was
confirmed, and in addition evidence was provided for Vβ restriction.

The significance of these findings is uncertain. Restricted TCR - Vβ usage might suggest initial activation by a superantigen-like activity as proposed in Type 1 diabetes (see section 1.2.3), but the evidence for this is weak in Davies’ study. The work of Quaratino et al. indicates that TCR Vα chain expression can influence antigenic specificity, but these studies also clearly show the serious pitfalls of inferring antigenic specificity from studies of TCR V-segment usage. Notably, McIntosh et al. have failed to show any evidence of restriction in Vα gene families in a separate study of thyroid lymphocytes obtained from patients with Graves’ disease, casting doubt on the initial observations [142,143].

### 1.3.2.4. Immune properties of thyroid epithelial cells

In thyroids of all species, the general metabolism of the thyroid epithelial cells -TECs (or thyrocytes, or follicular cells), their specialised functions and their growth are stimulated by thyrotropin [144,145]. TECs are stimulated by agonist α-TSH-R Abs to produce T4. However, TEC also have many properties which may enable them to play a role in the generation of the autoimmune response. These will be described below and the possible pathological consequences of these properties will be discussed in the next section.

#### (4a) MHC class II molecule expression

On normal human thyroid epithelial cells, only trace amounts of Class I (HLA-A, -B, -C) antigens have been found [146]. Class II antigens have not been detected [147]. However, the epithelial cells from the thyroid gland of patients with Graves’ disease display an increased density of Class I antigens and do express Class II antigens [147], which may confer on TEC the ability to activate CD4+ T cells[22].

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Aberrant Class II expression on TECs correlates with the titre of serum thyroid autoantibodies\textsuperscript{146}. It also correlates with the presence of infiltrating lymphocytes both in the gland as a whole\textsuperscript{146} and in different areas within the gland\textsuperscript{148}.

Early studies soon demonstrated that, IFN-$\gamma$, a T cell product, is the most powerful inducer of MHC class II expression on TECs\textsuperscript{147}. In addition, IFN-$\gamma$ induces HLA-DR:DQ:DP subregion expression in the same ratio \textit{in vitro}, as that observed on TECs \textit{in vivo}\textsuperscript{149}. This observation, along with the spatial and quantitative correlation between lymphocytic infiltration and MHC class II expression by TECs, suggests that such aberrant Class II expression \textit{in vivo} is not a primary event, but secondary to cytokine release by infiltrating lymphocytes. Strong supporting evidence for this is provided by the observation that transfer of Graves’ tissues into SCID mice followed by immunosuppressive therapy with FK506 results in disappearance of the lymphocytic infiltrate from the thyroid gland and, concomitantly, class II expression by the TECs.

\textbf{(4b) Adhesion molecules}

In addition to HLA Class II molecule expression, the expression of adhesion molecules required for interaction with T cells has also been studied in thyroid autoimmunity. TECs have been shown to express high levels of intercellular adhesion molecule-1 (ICAM-1)\textsuperscript{150-152} which binds lymphocyte function associated antigen 1 (LFA-1) on lymphocytes\textsuperscript{153}, and LFA-3\textsuperscript{151} which binds the lymphocyte surface antigen CD2\textsuperscript{153}. IFN-$\gamma$, TNF-$\alpha$ and interleukin-1$\alpha$ (IL-1$\alpha$) all increase the expression of ICAM-1 on cultured TECs \textit{in vitro}\textsuperscript{150,151} irrespective of whether TECs were derived from normal or autoimmune thyroid tissue\textsuperscript{151}. Unlike HLA Class II, ICAM-1 over-expression by TECs \textit{in vivo} seems specific for autoimmune conditions of the thyroid, since it is not seen in non-toxic goitre or carcinoma\textsuperscript{150-152}. 

\hspace{1cm} 

\textit{Anastasia Pappa} 

\textit{PhD Thesis}
(4c) Cytokine expression by TECs

Along with membrane molecules required for T cell interaction, cells of the target organ in autoimmunity may also synthesise and express soluble mediators able to affect lymphocyte function. TECs produce IL-1α and IL-6 but not IL-1β as shown by several different techniques: cytokine mRNA detection in tissue extracts \(^{[154]}\), immunostaining for cytokine protein on tissue sections \(^{[155,156]}\), analysis of supernatants from cultured TECs \(^{[155,156]}\) and, most definitely, by in situ hybridisation \(^{[155,156]}\). These cytokines are thought to play a part in inducing immunological changes in TECs including enhancement of both MHC Class I and Class II molecule expression, and upregulation of adhesion and complement regulatory molecule expression and may thus act in a cascade to enhance the autoimmune process.

(4d) Expression of co-stimulatory molecules

However, TECs do not express B7 (B7-1, CD80) \(^{[157-160]}\) or B7-2 (CD86) \(^{[158,161]}\), which play a more important role in T cell co-stimulation \(^{[162]}\). The CD40 molecule, which plays a crucial role in the reciprocal two-way communication between T cells and B cells, has been identified by means of immunohistochemistry to be expressed in human thyroid, but the cells responsible for this have not been identified \(^{[161]}\). CD40 expression has also been reported on TECs \(^{[163]}\) and more recently CD40 expression has been reported on TECs juxtaposed to lymphoid infiltrates \(^{[160]}\). Lately, Metcalfe et al. have shown by immunohistochemistry and flow cytometry that CD40 is expressed by TECs in vivo and in vitro in both autoimmune and non-autoimmune glands. CD40 expression was up-regulated by IL-1α and IFN-γ, but not by TSH. Although there was no significant effect of CD40 ligation on cAMP synthesis or \(^{[3]}H\)thymidine incorporation, there was no significant increase in IL-6 release by TECs. Thus, although TECs do not express members of
the B7 family of T cell co-stimulators, they do express CD40, indicating the possibility of mutually stimulatory T cell-TEC interaction \[^{164}\].

In conclusion TECs have been shown to have many features suggesting that they could act as antigen presenting cells.

**1.3.2.5. Possible role of thyroid epithelial cells as antigen presenting cells (mechanism of T cell activation).**

The mechanisms by which autoantigen reactive T cells become activated in Graves’ disease, as in other diseases (see section 1.2.3), is unclear. There are currently no reports of superantigen like activity in the thyroid gland, but cross-reactivity, at least at the antibody level with microbes such as Yersinia provides some evidence for molecular mimicry as a triggering event (see section 1.2.4). Following the observation of MHC class II molecules on TECs in 1983, Bottazzo and Feldmann proposed a role for endogenous presentation of autoantigens by TECs themselves in autoimmune thyroiditis \[^{22}\]. Given the wide range of immune function associated molecules expressed by TECs as detailed above, this is certainly a possibility and would explain both the epitope spreading to thyroid antigens (not just the T cell receptor) and the perpetuation of disease activity over time.

The most direct evidence that TECs can present antigen comes from the early studies of Londei \[^{27}\] showing that TECs can present viral antigens to cloned human T cells and later observations of the same group showing that TECs can stimulate class II restricted proliferation of thyroid-derived T cells \[^{165}\] and restimulate thyroid antigen-specific T cell clones derived from a patient with Graves’ disease \[^{3}\].

In further studies by Feldmann’s group, thyroid infiltrating T cells were also found to respond to cloned thyroid antigens (TPO, TSH-receptor), presented endogenously by EBV-transformed B cells \[^{132,133}\]. Interestingly, however, one T cell
derived cloned failed to respond to autologous thyroid epithelial cells and to recognise TPO expressed endogenously in EBV-transformed B cells, but was able to respond to exogenous whole TPO added to professional antigen presenting cells. Since all clones were derived from the site of disease and hence were likely to have been activated in vivo, this suggests that more than one antigen presenting mechanism is involved in activating TPO reactive T cells i.e. that professional as well as TEC-mediated antigen presentation may be occurring in Graves’ disease.

In contrast to these findings, suppression of IL-2 production and induction of anergy has recently been reported following antigen presentation by TECs derived from Graves’ disease to B7-dependent cloned human T cells. TECs were also found to promote IL-4 production in B7-independent T cell clones. The discrepancy in these reports remains to be explained, but may relate to differences in the responder T cell population which was not thyroid-derived in the latter studies.

These studies all examined the antigen presenting capacity of TECs derived from autoimmune disease. In the study of Grubeck-Loebenstein et al., TECs from non-toxic goitre failed to stimulate T cell proliferation even after exposure to IFN-γ in order to induce MHC class II expression. A similar failure of TECs to present antigen was seen with murine epithelial cells. The observation that transgenic islet cells expressing MHC class II can induce T cell anergy and that antigen presentation by other non-professional antigen presenting cells e.g. keratinocytes, may have similar effects, suggested a possible role for healthy TECs in down-regulating autoimmune responses. Such a role has been proposed but has yet to be confirmed. As discussed in section 1.3.2.4, TECs express some, but not all elements of professional antigen presenting cells. In particular, the lack expression of B7 molecules which in the study by Lechler’s group appeared to be responsible for a
tolerogenic outcome from antigen presentation.

### 1.3.2.6. Role of IFN-γ in the immunology of Graves’ disease

A central element of the hypothesis proposed by Bottazzo and Feldmann in 1983 involved a role for IFN-γ produced by infiltrating T cells in inducing aberrant MHC class II expression by TECs and hence autoimmunity. This proposal was supported by the finding that transgenic mice expressing IFN-γ by their islet β-cells develop diabetes. Furthermore, IFN-γ is almost certainly expressed in the thyroid in Graves’ disease. Although, histological and thyroid explant data do indicate that MHC class II expression by TECs is dependent on the presence of a T cell infiltrate, and hence presumably T cell-derived cytokines; clinically, treatment with IFN-α not IFN-γ results in thyroid autoimmunity.

Doubt on the central role of IFN-γ has also recently been cast by animal studies in which diseases can still be induced in animals with a disrupted IFN-γ receptor gene and in some cases accelerated disease results. Furthermore, IFN-γ treatment of DP-BB rats reduces the incidence of disease. Thus the role of IFN-γ and possibly involvement of other T cell products in modulating antigen presentation by TEC and promoting autoimmunity remains to be resolved.

### 1.3.2.7. The Th1/Th2 paradigm in Graves’ disease

The thyroid infiltrate in Graves’ disease contains CD4+ and CD8+ cells with many showing evidence of activation. Earlier studies suggested that Graves’ disease (GD)-derived CD4+ T cell clones produce a mixed Th1/Th2 cytokine pattern. Later studies using RT-PCR have detected IL-10 and IL-12 in thyroid infiltrates but IL-4 detection is variable.
Feldmann’s group have used an alternative approach. Using EBV-transformed B cell lines transfected with TPO or TSH-R as antigen presenting cells, they have demonstrated that GD-derived TPO-specific T cell clones have Th1 characteristics, whereas Th2 cells predominate in TSH-R-specific T cell clones. This may explain some of the discrepancies seen in studies of whole thyroid infiltrates and is consistent with a predominantly Th2-like response in cells involved in the pathogenesis of Graves’ disease as might be expected.

1.3.3 AUTOIMMUNE HYPOTHYROIDISM

Autoimmune hypothyroidism is a relatively common disease affecting 1% of women but no more than 0.1% of men. It is generally divided into goitrous (Hashimoto’s disease; HT) affecting middle aged women more often, and non-goitrous (primary myxoedema; PM) affecting the elderly mainly. Up to 5% of TAO cases are found in association with hypothyroidism. It is therefore likely that the cross-reactive mechanism that links TAO and thyroid disease is distinct from the mechanism causing thyrotoxicosis. However, TSH receptor antibodies that block rather than stimulate the receptor are detected in 20% of cases of autoimmune hypothyroidism and therefore this antigen may still represent the basis of cross reactivity with TAO. The role of the TSH-R as an antigen in TAO is discussed further in section 1.4.3 (page 57).

1.3.4 CLINICAL RELATIONSHIP OF TAO TO ATD

TAO has a variable but close temporal relationship with Graves’ thyroid disease (diffuse goitre with hyperthyroidism). In 1983, Gorman demonstrated that over 80% of patients with severe TAO develop eye findings within 18 months before or after the discovery of systemic thyroid disease. Prior to Gorman,
Amino et al. [185] showed in Japanese patients that there is a normal Gaussian distribution of exophthalmos in Graves' disease, suggesting concordance with hyperthyroidism. Similar data have been demonstrated in a Dutch study of 90 untreated patients with TAO [186] . The authors of the latter study noted that the age, sex and NOSPECS* distribution of both TAO and euthyroid Graves' disease patients at their institution were nearly identical, suggesting that they belong to the same population [186] . These and other considerations [187] have led some to assume that a common pathogenetic factor is present in TAO and ATD [188,189] . Others, however, are of the opinion that these disorders although closely related are distinct, because neither the severity nor the course of ophthalmopathy appear to correspond with the course of hyper- or hypo-thyroidism [190] .

The onset of eye disease usually coincides with that of Graves’ disease, however, one can precede the other by several years [183,191] . About 90% of patients with TAO also have Graves’ disease, the remaining 10% having Hashimoto’s thyroiditis or a subclinical form of thyroid autoimmunity in roughly equal numbers [192] . Of all the patients with Graves’ disease some 50% will have clinical evidence of ophthalmopathy, usually mild (Fig. 1.1, next page). Subtle signs of eye disease (raised intraocular pressure) or extraocular muscle (EOM) enlargement only detectable by imaging techniques are present in the majority [193] , if not in all, of the remainder [194,195] . Eye findings can occur in patients who have neither Graves’ disease nor evidence of thyroid gland inflammation. Similarly, thyroid-stimulating antibody levels, which often correlate with thyroid disease status and prognosis, do not correlate with eye findings [196,197] . While some patients with euthyroid TAO

* The NOSPECS system is a generally accepted classification of the most important signs of TAO [193] .
develop hyperthyroidism, a significant percentage do not. Finally, if ophthalmopathy is a feature of thyroid disease, it is puzzling that the eye disease is often asymmetric and has a predilection for some rectus muscles and not others \cite{196}, although careful imaging analysis can demonstrate that most, if not all, EOMs are involved in TAO \cite{194,195,198-205}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image}
\caption{This patient has lid retraction and fullness of upper lids with recent onset hyperthyroidism.}
\end{figure}
1.4 THYROID-ASSOCIATED OPHTHALMOPATHY - CLINICAL ASPECTS

1.4.1 Nomenclature of TAO

Several terms have been used to describe the ophthalmopathy that often accompanies hyperthyroidism and diffuse goitre (Table 1.2) and this reflects our poor understanding of the cause of the disease.

Table 1.2 Synonyms used to describe TAO*

<table>
<thead>
<tr>
<th>Name of Ophthalmopathy</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graves' ophthalmopathy</td>
<td>Thyroid exophthalmos</td>
</tr>
<tr>
<td>von Basedow's ophthalmopathy</td>
<td>Exophthalmic ophthalmoplegia</td>
</tr>
<tr>
<td>Dysthyroid ophthalmopathy</td>
<td>Exophthalmic ophthalmopathy</td>
</tr>
<tr>
<td>Endocrine ophthalmopathy</td>
<td>Thyrotrop(h)ic exophthalmos</td>
</tr>
<tr>
<td>Thyroid ophthalmopathy</td>
<td>Progressive exophthalmos</td>
</tr>
<tr>
<td>Thyroid ocular myopathy</td>
<td>Autoimmune orbitopathy</td>
</tr>
<tr>
<td>Endocrine exophthalmos</td>
<td>Endocrine orbitopathy</td>
</tr>
<tr>
<td>Endocrine eye disease</td>
<td>Dysthyroid orbitopathy</td>
</tr>
<tr>
<td>Thyroid eye disease</td>
<td>Ophthalmic Graves' disease</td>
</tr>
<tr>
<td>Malignant exophthalmos</td>
<td>Endocrine periorbitopathy</td>
</tr>
</tbody>
</table>

* Modified from P. Perros & P. Kendall-Taylor [206]

Some of the nomenclature is historically inaccurate (it was Parry who first described it [79] in 1786, not Graves [207], or von Basedow [208]) or misleading ("malignant" exophthalmos). Therefore a more appropriate term, at least for the present, would be "thyroid-associated ophthalmopathy" (TAO), which conveys two important messages, namely that the ophthalmopathy invariably occurs against the background of thyroid autoimmunity [209], and that it can occur in association with hyper-, hypo- or eu-thyroidism.
1.4.2 Epidemiology of TAO

The mean age of onset is 45 years (similar to Graves' disease). The female to male ratio is 2:1\(^{[191]}\), unlike Graves' disease which is 5-7:1. The prevalence of TAO in the U.K. is estimated to be 100 000-200 000, and of these some 5000-10 000 patients have severe eye disease\(^{[210]}\). After its onset, the disease may progress to reach its peak of severity, then levels, and gradually improves, though return to normality is unusual\(^{[64,211-213]}\).

The severity of TAO increases with advancing age, especially in males\(^{[214,215]}\). Thyroid antibodies or abnormal TSH levels are found in many euthyroid TAO patients. One recent study of 21 euthyroid TAO patients found evidence for autoimmune involvement of the thyroid in all cases, although some of the assays used remain to be fully validated\(^{[209]}\). Treatment of Graves' disease with anti-thyroid drugs or subtotal thyroidectomy appears to have little, if any, effect on TAO but radioiodine may double the risk of eye signs developing or worsening\(^{[216]}\). The reasons for this are not known, although it may be important that thyrotoxicosis persisted after radioiodine in many of the patients who experienced eye problems.

Smoking has been strongly associated with TAO, with both the prevalence and severity of signs increasing with cigarette consumption\(^{[217-220]}\). Smoking is also a risk factor in Asian Indians with Graves' disease, despite their low prevalence of TAO. This association may explain why TAO is more frequent in elderly men, as these had the highest exposure to tobacco. However, Pfeilschifter's and Ziegler's recent data\(^{[220]}\) suggest that current, but not lifetime, tobacco consumption constitutes a risk for the incidence of proptosis and diplopia in patients with Graves' hyperthyroidism, and that this risk increases up to 3.1-fold with smoking severity.
1.4.3 Anatomical considerations in TAO

The orbit is a pear-shaped bony socket that contains the globe of the eye, its supporting muscles, nerves, blood vessels, orbital fat, and the lacrimal gland. Since these tissues are contained within a confined space, any significant increase in their volume may result in anterior globe displacement (proptosis). In TAO (Fig. 1.2, Fig. 1.3), an increase in the volume of the extraocular muscles and retrobulbar connective tissue displaces the globe anteriorly. Therefore, from a purely mechanical standpoint, most of the signs and symptoms of TAO can be understood.

Fig. 1.2 Unilateral TAO affecting right eye which is displaced forwards and downwards.

Fig. 1.3 Bilateral long term changes with “burnt-out” TAO with persistent proptosis and lid retraction.
1.4.4 Symptoms and signs

These may include lid retraction, soft tissue changes, proptosis, disturbances in ocular motility, diplopia, corneal lesions and loss of sight[^222,223].

(4a) Lid retraction

In most patients with TAO, the eye signs are confined to mild upper lid retraction and lid lag in downward gaze, two of the earliest signs of TAO[^221], (Fig. 1.4). Although the exact mechanism causing these signs is not known, swelling of the superior rectus-levator muscle complex has been held responsible. Thyrotoxicosis, by increasing sympathetic tone can cause lid retraction and even some proptosis, which can be relieved by cervical sympathectomy[^224,225]. However, this is seen more frequently in Graves' disease than in other forms of thyrotoxicosis[^226]. Apart from the slight inflammation of the levator muscle[^227], many adhesions to the surrounding tissues are found[^228], which may be important in causing the upper lid retraction. Lower lid retraction is also frequently present[^229], contributing also to the wide lid aperture.

(4b) Soft tissue involvement

Puffy, swollen lids, conjunctival injection (especially over the four rectus muscles, Fig. 1.5, next page) and chemosis are generally ascribed to impaired venous drainage due to the enlarged orbital tissues and to an increase in orbital pressure[^222,230,231]. This concept is in keeping with the observed decrease in

chemosis after lowering orbital pressure by transantral decompression\textsuperscript{[232]}. Swelling of the lids may also result from herniation of retrobulbar fat through hiatuses in the orbital septum\textsuperscript{[233-236]}. In patients with active TAO, the inflamed lids appear oedematous and a diffuse redness can be discerned\textsuperscript{[237]}. This may indicate actual subcutaneous inflammation\textsuperscript{[234]}. However, it is clinically hard to distinguish between these different forms of lid inflammation.

\textbf{(4c) Proptosis / Exophthalmos}\textsuperscript{*}

To date, it has become clear that the enlarged retrobulbar tissues, confined within the orbital walls, have no other outlet than pushing the globe forwards\textsuperscript{[238]}. In the past, however, proptosis was thought to result\textsuperscript{[239]} from venous obstruction at the level of the thyroid gland and sympathetic overactivity\textsuperscript{[240]} (Fig. 1.6). Meticulous postmortem studies\textsuperscript{[241]} demonstrated that the normal volume of the EOMs is 3.5-4 ml and of the orbital cavity only 26 ml. An increase of 4 ml in EOM volume produced

\textsuperscript{*} These terms may be used interchangeably since the artificial separation in Duke-Elder's System of Ophthalmology can no longer be supported\textsuperscript{[239]}.
6mm of proptosis. Therefore, changes in EOM volume can cause considerable exophthalmos. The increase in EOM volume can be pronounced and muscles 3-8 times the normal size have been encountered \(^{[242]}\). Thus, exophthalmos is the result of an increase in retrobulbar tissues and can be viewed as “nature’s decompression” \(^{[243]}\) which can also be seen on the orbital scans as inward bowing of the ethmoidal sinuses by enlarged medial recti - “the Coca-Cola bottle sign” (Fig. 1.7).

(4d) Ocular motility disturbances

Vertical diplopia is not due to superior rectus ophthalmoplegia \(^{[233]}\), as was believed for many years, but to tight, fibrosed EOMs that do not extend fully when their antagonist contracts. If impairment is asymmetrical, the patient will have double vision, a major source of morbidity \(^{[244]}\). Fibrosis of the inferior rectus, the most frequently affected muscle \(^{[202,245]}\), causes limitation of elevation \(^{[234]}\) (Fig. 1.8) and diplopia on
Chapter 1: Introduction

attempts upgaze, and the agonist -the superior rectus muscle- does not show electromyographic signs of paresis \[^{246}\]. Additional evidence of restricted elevation is provided by measurement of the intraocular pressure (IOP) in the primary position and then on attempted upgaze, when the IOP may increase by 6 mm Hg or more proving that superior rectus is contracting \[^{223}\]. In view of the age range of these patients (25-78 years), surprisingly few have open angle glaucoma as well.

Disturbed ocular movements can be incapacitating by causing diplopia, especially in the primary and reading position. If impairment is exactly symmetrical \[^{247}\], or has become so by adopting an abnormal head position (ocular torticollis), no diplopia will be noticed \[^{234}\].

**4(e) Corneal lesions**

Exophthalmos, lid retraction, stare, and less frequent blinking; all contribute to the exposure of the cornea (Fig. 1.9) and in later stages can lead to corneal ulceration, infection, and endophthalmitis \[^{223}\] (Fig. 1.10, next page).

**4(f) Optic nerve involvement**

There is no evidence for direct inflammation of the optic nerve \[^{222,234}\]. Optic neuropathy is thought to be secondary to EOM enlargement \[^{238,248}\] and a general increase in retrobulbar pressure \[^{249}\]. Optic neuropathy is thought to be caused by the pressure of the enlarged EOMs encircling the optic nerve at their site of attachment in the orbital apex ("apical crowding") as visualised on orbital computerised

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Fig. 1.9 Male TAO patient with gross unilateral proptosis and right eye fixed in downgaze. Corneal exposure is caused by inadequate lid closure.
tomography\textsuperscript{[250]} and magnetic resonance imaging\textsuperscript{[205]} scans. This is in accordance with the correlation found between total muscle volume and optic nerve involvement\textsuperscript{[235]}. The apparent paradox of severe eye disease and only moderate exophthalmos\textsuperscript{[236]} in some patients may be a result of their well developed and firm connective tissue containing the proptosis, resulting in high intraorbital pressure and optic nerve compression.

Sight loss due to optic nerve damage can be accompanied by impaired colour vision and visual field defects\textsuperscript{[251]}. In rapid onset or severe ("malignant") exophthalmos optic disc oedema is seen (Fig. 1.11), which with time may change to optic disc pallor with visual field defects although treatment can still restore vision\textsuperscript{[247,252]}.

\textbf{Fig. 1.10} End stage of severe corneal exposure with corneal ulceration and pus in the anterior chamber (hypopyon; arrowed).

\textbf{Fig. 1.11} Optic disc oedema
1.4.5 Orbital Imaging

The diagnosis of TAO can be made in most cases on clinical grounds; in 90% of Graves' patients subclinical TAO can be detected depending upon the measures used. In a few cases confirmation is required, particularly in patients with unilateral disease, and in patients with euthyroid eye disease (no previous or present thyroid dysfunction). The problem is resolved by imaging of the orbits using computerised scanning \cite{195} (Fig. 1.12, Fig. 1.13, (Fig. 1.14, next page)), magnetic resonance imaging scanning \cite{205} or (Fig. 1.15, next page) ultrasonography \cite{194}.

Enlargement of EOMs is usually bilateral in TAO and involves the bellies of several muscles with sparing of the muscle insertions \cite{253}. Recently, uptake of radiolabelled octreotide in the orbit has been reported, which may prove to be a useful new technique \cite{254}.

\textbf{Fig. 1.12} CT scan of a male patient with severe TAO showing hugely swollen EOMs.

\textbf{Fig. 1.13} CT scan of a patient with TAO. The globes are proptosed beyond the lateral orbital rim (marked by horizontal line), with normal size EOMs. In this case proptosis is due to excess retro-ocular fat/connective tissue.
Fig. 1.14  Horizontal orbital CT scan of a TAO patient showing a grossly enlarged left medial rectus (bottom). However, on the reformatted side view along the indicated axis through the right orbit, an equally enlarged right inferior rectus is observed (top).

Fig. 1.15  An oblique side view of an MRI scan from a TAO patient showing enlarged inferior rectus (IR), superior rectus (SR) and levator palpebrae (Lev P) muscle (as arrowed).
### Table 1.3 NO SPECS classification of ocular changes in TAO*

<table>
<thead>
<tr>
<th>Class</th>
<th>Grade</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>No signs or symptoms</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>Only signs</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>Soft tissue involvement with symptoms and</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>Minimal</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>Proptosis</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>&lt; 23 mm</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>23-24 mm</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>25-27 mm</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>Extraocular muscle involvement</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>Limitation of motion at extremes of gaze</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>Evident restriction of motion</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>Corneal involvement</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>Stippling of cornea</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>Ulceration</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>Sight loss (Visual acuity(^\S))</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>&gt; 0.67(^\S)</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.67-0.33</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>&lt; 0.32</td>
</tr>
</tbody>
</table>

* Modified slightly from the classification of Werner\(^{[193]}\)

\(^\S\) Visual acuity is given in decimal notation: e.g. 20/20 = 1.0; 20/30 = 0.67; 20/40 = 0.50; 6/6 = 1.0; 6/9 = 0.67; 6/12 = 0.50
1.4.6 Assessment of disease progress and activity

Detailed, careful examination is essential in documenting the severity of the disease and assessing its progress with time. Werner’s NO SPECS classification \(^{255}\) (Table 1.3), serves as a simple tool to memorise the various features of TAO offering a static measurement of the involvement of the different parts of the eye. In addition, not all signs and symptoms are present in every patient, and patients do not progress serially from one class into another \(^{193,256}\). The NO SPECS classification thus has been criticized \(^{256-259}\) and recent recommendations of an international working group emphasized the need for objective measurements, whenever possible, of the individual components of the disease and its activity \(^{260}\) (Fig. 1.16; (Fig. 1.2, page 57)). It was suggested that the components of the disease should be assessed separately, as they may be present to varying degrees in patients and be independent of each other in the way they progress or respond to treatment (Table 1.4, next page). The Clinical Activity Score (Table 1.5, page 68) is a novel means of assessing the “activity” of the disease, which has been evaluated and used to predict response to treatment \(^{237}\). Attempts to devise a Clinical Activity Score measurement are important as this can indicate that the patient requires prompt immunosuppressive therapy or even surgical decompression.

Fig. 1.16 Female patient with active TAO whose attempt to look to her right is limited by orbital pain.
### Table 1.4 Classification of ocular changes in TAO according to recent recommendations*

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lids</strong></td>
<td>Measure maximal vertical lid fissure and position of lids in relation to corneoscleral limbus</td>
</tr>
<tr>
<td><strong>Cornea</strong></td>
<td>Note presence or absence of exposure keratitis, or ulceration (examine with rose Bengal, and fluorescein drops)</td>
</tr>
<tr>
<td><strong>Extraocular Muscles</strong></td>
<td>Note presence or absence of single binocular vision with and without prisms in the central 30°. Chart ocular movements using Hess/Lees’ screen. Additional optional measurements include measurement of cross-sectional diameter of muscles by an imaging technique, and intraocular pressure measurements in different gaze directions</td>
</tr>
<tr>
<td><strong>Proptosis</strong></td>
<td>Exophthalmometer measurements of each eye separately fixating in the primary position</td>
</tr>
<tr>
<td><strong>Optic nerve</strong></td>
<td>Visual acuity, visual fields, and colour vision should be measured</td>
</tr>
<tr>
<td><strong>Clinical Activity Score</strong></td>
<td>In addition to the above parameters measured serially, the clinical activity score is calculated by assigning one point for the presence of each of the following: spontaneous retrobulbar pain, pain on eye movement, lid erythema, conjunctival injection, chemosis, swelling of the caruncle, and lid oedema or fullness</td>
</tr>
<tr>
<td><strong>Patient self-assessment</strong></td>
<td>Self-reported severity (described on scales of best to worst) of appearance, visual acuity, eye discomfort and diplopia</td>
</tr>
</tbody>
</table>

* Modified from reference number [260].
Table 1.5 The Clinical Activity Score classification of ocular changes in TAO\textsuperscript{[237]}

<table>
<thead>
<tr>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pain</strong></td>
</tr>
<tr>
<td>painful, oppressive feeling in or behind the globe,</td>
</tr>
<tr>
<td>pain on attempted up, side, or down gaze</td>
</tr>
<tr>
<td><strong>Redness</strong></td>
</tr>
<tr>
<td>redness of the lid(s), diffuse redness of the conjunctiva</td>
</tr>
<tr>
<td><strong>Swelling</strong></td>
</tr>
<tr>
<td>chemosis,</td>
</tr>
<tr>
<td>swollen caruncle</td>
</tr>
<tr>
<td>oedema of the lid(s)</td>
</tr>
<tr>
<td>increase of proptosis by 2 mm or more during a period of between 1 and 3 months</td>
</tr>
<tr>
<td><strong>Impaired function</strong></td>
</tr>
<tr>
<td>decrease in visual acuity of 1 or more lines on the Snellen chart (using a pinhole) during a period of between 1 and 3 months</td>
</tr>
<tr>
<td>decrease of eye movements in any direction equal to or more than 5 degrees during a period of between 1 and 3 months</td>
</tr>
</tbody>
</table>

For each of the signs present, one point is given.
The sum of these points defines the Activity Score.
Table 1.6 Current therapeutic management of TAO*

<table>
<thead>
<tr>
<th>Diagnosis of TAO</th>
<th>Hyperthyroid</th>
<th>Euthyroid</th>
<th>Hypothyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperthyroid</td>
<td>Carbimazole, Propylthiouracil, Radioiodine, sub-total thyroidectomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthyroid</td>
<td>Thyroxine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothyroid</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**maintain euthyroidism**

- Sight-threatening TAO
  - (1) i.v. Methylprednisolone, *if* no response
  - (2) Surgical decompression

- Active TAO
  - (1) Retrobulbar radiotherapy

- "Burnt-out" TAO
  - Disfiguring proptosis >26 mm?
    - No
    - Yes
      - Double vision & compensatory head posture
        - correctable by prisms?
          - Yes
            - Upper & Lower lid retraction?
              - Yes
                - Lid lengthening
              - No
                - Dermatochalasis?
                  - Yes
                    - Blepharoplasty
                  - No
                    - Follow-up
            - No
                - Strabismus surgery *(more than one operation may be needed)*

* (personal communication with Peter Fells)
1.4.7 Current therapeutic approaches

No single treatment plan is optimal for all patients with TAO. Many patients will not require intensive treatment (immunosuppression, or specialized surgery) and symptomatic measures will suffice (Table 1.6). As in many medical conditions the multiplicity of therapeutic remedies confirms our limited knowledge of the pathogenesis of TAO.

(7a) Medical management of TAO

Local measures dealing purely with the symptoms of dry eyes and photophobia may be tried.

For “active” TAO retro-ocular irradiation (Fig. 1.17), (the rationale of which rests on the premise that infiltrating lymphocytes mediate pathologic changes in EOM or retro-ocular connective tissue and that these lymphocytes, especially sensitised CD4+ T cells, are markedly radiosensitive), in a total of 2000 centiGrays in ten fractions over a period of 2 weeks has been reported to be effective in 2/3 of patients with Graves’ophthalmopathy with minimal risk of complication. Also, for “active” TAO systemic corticosteroids starting at 80 mgs a day and tapered slowly over 4 months or very high doses of methylprednisolone (pulses of 500 mg given intravenously) are effective in about 65% of patients (Fig. 1.18 a and b). These methods have been tried in

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*Fig. 1.17* Isodose curves of radiotherapy superimposed onto orbital CT scan of a patient with TAO. The lens (arrowed) in each eye receives less than 10% of the irradiation.
combination and serially. Treatments that have been used in the past, using Cyclosporine and plasmapheresis, are rarely used nowadays.

Two new methods under trial at present are the immunoglobulin treatment and that of octreotide. However, immunoglobulin therapy in a regime of intravenous infusion over two successive days as an in-patient, with the course repeated every 3 weeks for a total of 7 episodes is both time-consuming and very expensive with the additional risks of HIV or hepatitis transmission. Octreotide, a potent long-acting somatostatin analogue, has been used with some beneficial effects on TAO in some studies \cite{265-267}, but not confirmed by other studies \cite{268}.

**Fig. 1.18** Coronal cut orbital CT scans behind the eyeballs in the same TAO patient: (a) before and (b) after high dose corticosteroid therapy. All of the EOMs in the right orbit returned to normal size, however in the left orbit the medial and inferior recti stayed enlarged (arrows).

(7b) **Surgical management of TAO**

Surgery has a definite place in the treatment of “burnt out” TAO (Table 1.6, page 69). With the exception of progressive optic neuropathy not responding to conservative treatment, surgery is usually postponed until the active phase of the disease has resolved \cite{269}. For optimal effect, apart from the right time, surgical
interventions must be made in the right order as well for any particular patient [270-274].

The reasons for operating are: (1) threatened loss of vision from optic nerve compression or gross corneal exposure with ulceration and endophthalmitis; (2) severe proptosis with subluxation of the globe; (3) double vision or marked compensatory head posture; (4) excessive retraction of upper and/or lower lids; (5) cosmesis.

1.4.8 Natural history of TAO

The natural history of TAO is one of persistent proptosis, although the local inflammatory symptoms and signs usually subside gradually. The course is largely independent of the type of antithyroid therapy used. However, achievement of euthyroidism by any form of antithyroid treatment does seem important, as ophthalmopathy is more likely to persist or worsen in patients with ongoing thyroid dysfunction [275]. Most investigators agree that the disease is self-limiting and runs a course of exacerbations and spontaneous remissions within a period of between 3 months and 3 years [276,277]. Fewer than 10% of patients develop severe eye symptoms.

In a long-term (mean 5 years, range 1 to 11 years) retrospective study of 218 patients who had hyperthyroidism and TAO, in whom hyperthyroidism was treated with either (a) an antithyroid drug, (b) radioactive iodine, or (c) subtotal thyroidectomy, the ophthalmopathy progressed in approximately 20% of the patients in each treatment group [278]. It worsened enough to warrant glucocorticoid or surgical therapy in only 18 of the 218 patients, usually within two years after the initiation of antithyroid treatment. Conversely, ophthalmopathy improved in 12 to
14% of the patients in the three treatment groups. Among 288 hyperthyroid patients who initially had no clinically evident ophthalmopathy, approximately 6% in each treatment group developed it during the follow-up period. In another study of 122 patients followed for 3 to 19 years after therapy for hyperthyroidism, proptosis did not change in 79, worsened in 16%, and improved in 6% \([279]\). When progression occurs, it is relatively slow: the median duration of ophthalmopathy was 23 months among 163 previously hyperthyroid patients who underwent orbital decompression surgery at the Mayo Clinic \([183]\).
1.5 CURRENT UNDERSTANDING OF THE PATHOGENESIS OF TAO

1.5.1 Immunogenetics in TAO

Race is an important determinant of eye signs, with TAO being 6 times more common in Caucasians than in Asian Indians with Graves' disease living in the same region. Many patients report relatives who also suffered from hyperthyroidism and “large eyes”. However, attempts to find a genetic susceptibility locus have been largely unsuccessful. HLA-DR3 and -DR7 were weakly associated with an increased risk of TAO in Hungarian Graves’ disease patients, but there were no differences in HLA-DR or DQ alleles in two studies of Caucasian patients in the United Kingdom. HLA-DQP1*0201 appears to protect against TAO in Caucasians, and a similar effect was observed in Japanese patients. Although American Blacks with TAO had an increased frequency of HLA-DR4 and Drw6 compared with controls, there was no significant difference (after correction for the number of antigens tested) between patients with and without eye signs, suggesting again that there is no clear HLA difference.

In North East England, genetic associations of Graves' patients with and without TAO were studied in two patient populations. In this study, the authors concluded that although TAO shares the same HLA associations as hyperthyroidism, it differs in the increased frequency of P blood group, suggesting that additional genetic factors may determine which patients with Graves’ disease develop ophthalmopathy. However, others could not confirm these findings.
1.5.2 Histological findings in TAO

In 1886, Silcock[^286] first noted EOM changes in an autopsy study of a patient with TAO. He noted yellowish discoloration of the muscles with interfascicular fatty infiltration. Subsequently, a number of other authors have observed gross EOM changes in TAO: EOMs were 2-8 times enlarged, rubbery, dark red, oedematous, and fibrotic[^287,288] (Fig. 1.19). On examination they are stiff and lose their compliance. In less than 25% of patients with TAO an isolated increase in orbital fat/connective tissue volume (not in conjunction with enlarged EOM bodies) is observed by imaging techniques or at surgery[^289], (Fig 1.13, page 63).

Histologic examination of affected EOMs, and retro-ocular fat/connective

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**Fig. 1.19** (a) Post-mortem specimens of severe TAO demonstrating the grossly enlarged EOMs. (b) Exenterated specimens from a normal cadaver shown for comparison. (Reproduced from Levitt T: *The Thyroid*. Churchill Livingstone, Edinburgh, 1954, with the permission of the publishers).
tissue specimens from patients with severe, active TAO, commonly reveals an interstitial inflammatory oedema, with excess collagen and hydrophilic glycosaminoglycans, predominantly hyaluronic acid. The inflammatory infiltrate consists of activated T cells, macrophages, with some mast cells and plasma cells as shown by immunohistochemical studies. Both helper/inducer (CD4^+) and suppressor/cytotoxic (CD8^+) T cells have been shown to be present in the infiltrate. The ratio of CD4^+:CD8^+ infiltrating T cells on frozen EOM biopsies was at least 2:1, whereas in retro-ocular fat/connective tissue specimens a variable predominance of CD8^+ T cells has been reported. A significant proportion of T cells, frequently detected adjacent to blood vessels, has been demonstrated to be CD3^+/CD45RO^+ cells, reflecting a subset of memory T cells in addition to increased numbers of macrophages. Immunohistochemical characterisation of the T cell antigen receptors expressed in situ in TAO has revealed that the majority of retro-ocular T cells belong to the α/β phenotype.

Although the EOM tissue may be grossly enlarged in TAO during the active inflammatory stage of the disease, the muscle fibres themselves are morphologically intact. Any alterations of muscle fibres are probably a secondary phenomenon, due to chronic compression and inflammation of surrounding connective tissue. This surprising finding that EOM fibres are intact in TAO has focused attention on other cells in the EOM tissue and retro-ocular fat/connective tissue as candidate target cells primarily affected in this disease.

The connective tissue surrounding muscle fibres also contains increased numbers of fibroblasts and collagen. Some fibroblast-like cells have been shown to express spontaneously HLA class II molecules. However, this was also noted in orbital fibroblasts of unaffected normal subjects. By
immunohistochemistry, Weetman et al. reported that 2 of 3 patients with TAO showed HLA-DR expression on the interstitial cells in EOM tissue, but not on the eye muscle fibres \[^{290}\]. Tallstedt and Norberg also tested 5 patients, including 1 subject who had not received immunosuppressive treatment before surgery, for HLA-DR expression in EOM tissue. They found HLA-DR expression on the interstitial cells, especially capillary endothelial cells, from both patients with TAO and normal subjects \[^{296}\]. Heufelder and Bahn reported positive immunostaining for HLA-DR, detected on fibroblasts forming the perimysial connective tissue that separates and surrounds EOM fibres, on EOM tissue obtained at autopsy from one patient with severe TAO \[^{291}\]. In contrast to these studies, Hiromatsu et al. reported in 1995 \[^{298}\], also by immunohistochemical staining on frozen EOM tissue sections obtained from patients with active TAO, HLA-DR expression on EOM fibres (albeit in only 4 of the 38 cases studied) in association with, in all cases, moderate to marked lymphocytic infiltration.

In summary, several reports have demonstrated expression of HLA Class II molecules on EOM fibroblasts in retro-ocular tissue by immunohistochemistry. Only in one report was HLA class II expression described on the EOM fibres themselves \[^{291}\]. However, this only occurred in 4 of the 38 cases studied and only in association with moderate or marked lymphocytic infiltration. HLA-DR expression appears to be present on capillary endothelial cells in both patients with TAO and normal subjects but expression on fibroblasts is confined to TAO itself.

The significance of HLA-DR expression by retro-ocular fibroblasts in TAO remains unclear. By analogy with other cell types (e.g. thyroid epithelial cells \[^{299}\]) HLA-DR expression is induced by exposure to interferon-\(\gamma\) (IFN-\(\gamma\), secreted by infiltrating T cells. Similar effects have been demonstrated in vitro with retro-ocular
fibroblasts, and expression in TAO is therefore likely to reflect cytokine release by infiltrating T cells. In ATD HLA-DR expression on thyroid epithelial cells has been proposed to present antigen to T cells fuelling the autoimmune process. A protective role has also been proposed. In all likelihood HLA-DR expression by retro-ocular fibroblasts in TAO is secondary to cytokines secreted by the inflammatory infiltrate in affected tissues. However, the role of expression of these molecules in TAO remains to be identified. Even if retro-ocular fibroblasts are not involved in antigen presentation in TAO, changes in their biosynthetic activity (e.g. secretion of GAGs) are considered to be a key element in the gross pathological changes of TAO.

1.5.3 Role of autoantigens in TAO - relationship with ATD

Unlike autoimmune thyroid disease in which a number of autoantigens have been identified and characterised, the identity and nature of the target antigen(s) in TAO is far from clear. Over the years a number of candidate eye antigens and links with the thyroid have been proposed and are as described below.

(3a) EOMs as a source of autoantigens

Cross-reacting antibodies to epitopes common to EOMs and thyroid, but not present in skeletal muscle, have been proposed to explain the pathogenesis of TAO and its link with ATD. A number of techniques have been employed by different investigators to identify an autoantigen cross-reacting between the thyroid and the orbit. Earlier studies have included enzyme-linked immunoassays, immunocytochemistry, antibody-dependent cytotoxicity assays, investigations of antigen specific T cell proliferation, and also three kinds of species (human, bovine, porcine) as source of EOMs, different methods of
preparation of the antigenic substrate, as well as different groups of TAO patients \([301,302,306-314]\). Not surprisingly, the reported results were heterogeneous. In addition, Western blotting analysis has failed to clarify the nature of the autoantigens \([189,308,315]\). Using murine monoclonal antibodies raised to eye muscle membranes, a variety of muscle antigens have been identified which appeared also to be present in thyroid tissue but were subsequently also found in skeletal muscle and other tissues \([316,317]\). An alternative approach eliciting the biological activity of EOM antibodies on muscle fibres was reported in 1992 \([318]\). Using cloned porcine extraocular myoblasts, it was demonstrated that IgG from patients with TAO stimulated proliferation of the myoblasts \textit{in vitro} in a dose-related fashion compared to normal control IgG \([318]\). IgG from TAO patients evoked greater growth responses than IgG from controls or from Graves' disease patients without TAO. This growth-stimulating activity correlated with binding activity to muscle membranes suggesting that this mechanism may contribute to EOM enlargement in TAO.

\textbf{(3b) The 64 kDa autoantigen}

In 1988, Wall and colleagues reported the identification of a 64 kDa protein in the membrane fraction of human EOM, orbital connective tissue and thyroid, by testing sera of patients with TAO in SDS-polyacrylamide gel electrophoresis and Western blotting \([319]\), and proposed to be a thyroid/orbit-shared antigen, thus developing the hypothesis of cross-reactivity between TAO and ATD \([187]\). Subsequently, Vassart's group screening a thyroid expression library with ATD sera cloned and sequenced a 64 kDa protein (D1) which had a limited tissue distribution \([320]\). Sera from patients with TAO were shown to bind to the cloned 64 kDa protein \([321]\) but using polymerase chain reaction (PCR), the tissue distribution was found to be more widespread than in the earlier Western blot \([322]\). 64 kDa
reactivity was also found in non-TAO sera\textsuperscript{[189,315]} Thus, D1 is unlikely to be relevant to the pathogenesis of TAO but it is possible that another antigen of 64 kDa is implicated.

\textbf{(3c) The thyroglobulin (Tg) / acetylcholinesterase-shared epitope}

Comparison of the nucleotide and peptide sequences of thyroglobulin and acetylcholinesterase (ACHE) suggested that they might have shared epitopes\textsuperscript{[323]} and that this could provide a link between the thyroid and the orbit\textsuperscript{[324]}. Screening a thyroid expression library with anti-ACHE antibody a shared epitope was identified corresponding to residues 2376-2464 of Tg and residues 204-292 of ACHE\textsuperscript{[325]}. Subsequent tests of larger numbers of Graves’ and TAO patient sera showed that reactivity with this fragment of Tg did not always correlate with TAO severity\textsuperscript{[326]}. Therefore the role of this epitope in the pathogenesis of TAO remains unclear.
(3d) The TSH-R and TSH-R antibodies

Thyrotropin exerts its biological effects by binding to the TSH-R on the thyroid cell plasma membrane \(^{[327,328]}\) followed by activation of the adenylate cyclase-cAMP pathway \(^{[329,330]}\) and also, although less well defined, the phosphatidyl inositol \(^{[331]}\) pathway. The serum component responsible for thyrotoxicosis in Graves’ disease \(^{[124]}\), identified to be an immunoglobulin in 1964 \(^{[126,332]}\), is a TSH-R autoantibody (TSH-R Ab) \(^{[333,334]}\). Thus, elucidation of the structure and function of the TSH-R has been a very important element in recent studies of Graves’ disease owing to its physiological importance and its implications in this disease.

The cloning of the TSH-R was initially reported from the laboratory of Vassart from Brussels in 1989 using a canine thyroid library \(^{[335]}\). Thereafter, several groups using different cloning techniques reported the sequence of the TSH-R from human \(^{[128,336-338]}\) and \(^{[339]}\) rat thyroid tissue.

(3d1) The extrathyroidal thyrotropin receptor - possible role in TAO

A small percentage (less than 10%) of patients with Graves’ disease have also pretibial dermopathy, and even more rarely associated finger and toe acropachy may be seen \(^{[63]}\). Patients displaying one or more of these manifestations generally also develop severe TAO, with high concentrations of TSH-R stimulating immunoglobulins almost invariably being detectable in their sera \(^{[340]}\). If TSH-R is expressed on orbital and pretibial fibroblasts membranes, then it could be recognised by circulating T cells in conjunction with HLA molecules or activated by thyrotropin-receptor antibodies. This might then explain the close clinical association among Graves’ hyperthyroidism, ophthalmopathy, and pretibial dermopathy. Furthermore, the less frequent association of TAO with Hashimoto’s thyroiditis could be explained on the basis of such cross-reactivity. \(^{[341]}\)

Anastasia Pappa PhD Thesis
Fig. 1.20 Schematic representation of the human TSH receptor. The seven transmembrane segments characteristic of the G protein-coupled receptors are shown together with the long amino-terminal extracellular domain (Y symbols indicate the positions of N-glycosylation acceptor sites) and the carboxy-terminal intracellular tail. The natural mutations responsible for hyperfunctional thyroid adenomas (Ala-623→Ile, Asp-619→Gly, [341]) and hereditary non-autoimmune toxic thyroid hyperplasia (Val-509→Ala, Cys-672→Tyr, [342]) are indicated. (Reproduced with permission from reference number [343], copyright © 1995, by Baillière Tindall).
TSH-R is a member of the G protein-coupled receptor family characterised by 7 transmembrane sequences \cite{128,336-338}. The protein is organized into 3 distinct regions: (a) an extracellular domain, followed by (b) a transmembrane spanning domain, and (c) an intracellular carboxy-terminal domain (Fig. 1.20). Analysis of the amino acid sequence, deduced from the nucleotide sequence of its cDNA, reveals a polypeptide of 744 residues, 398 forming the extracellular domain (ECD) having 6 potential \(N\)-linked glycosylation sites and 346 residues comprised of seven hydrophobic regions which span the membrane, a feature common to G protein-coupled receptors. A feature of the ECD is the loose repetition of a motif of 25 residues rich in leucine which is also found in a number of widely different proteins and seems to confer the ability to interact with other proteins \cite{128,335}.

The human TSH-R is encoded by a single gene that spans over 60 kilobases \cite{344} and is located on chromosome 14q31 \cite{344}. The receptor has been shown, by immunoblot studies and immunoaffinity purification, to be composed of 2 subunits linked by disulphide bridges and probably derived by proteolytic cleavage of a single 90-kDa precursor \cite{345}. The extracellular \(\alpha\)-subunit (hormone binding), which is glycosylated, has been reported to have an apparent molecular mass of 53 kDa, whereas the \(\beta\)-subunit, which contains both the transmembrane and intracellular domains of the receptor, has been reported to have an apparent molecular mass of 33-42 kDa \cite{345}.

The mature receptor (84 kDa before glycosylation) is a glycoprotein with intramolecular disulphide bonds linking the highly conserved clusters of cysteine residues located at the amino and carboxy termini of the extracellular domain.
Proteolytic cleavage releases a TSH binding fragment of about 54 kDa. The extracellular domain contains at least 6 potential glycosylation sites \[^{346}\]. With the cloning of the TSH-R, TSH-R transcripts or cDNA fragments have been detected in human peripheral blood lymphocytes \[^{347}\], rat adipose and retro-ocular tissues \[^{348}\], guinea pig white and brown adipose tissue \[^{349}\] as well as in human retro-ocular tissues \[^{348,350-354}\] although the results of the latter studies are unfortunately conflicting. The protein structure of the receptors expressed in these tissues has not been established yet.

Prior to the molecular cloning, sequencing, and expression of the cDNA of the human TSH-R \[^{335,336,344}\], some evidence had been provided in favour of this antigen (TSH-R) existing in tissues outside of the thyroid gland, including retro-ocular tissue and adipocytes using guinea-pig retro-ocular tissue plasma membranes and solubilised guinea-pig fat cell membranes respectively \[^{355,356}\]. Reversible low-affinity binding sites for \(^{125}\)I-labeled bovine TSH had been detected on porcine orbital connective tissue membranes, and found to be preferentially recognised by IgGs from patients with TAO \[^{357,358}\], although, an earlier study had failed to detect binding of radiolabelled TSH to human retro-ocular fat membranes \[^{359}\].


<table>
<thead>
<tr>
<th>Study</th>
<th>Reference</th>
<th>Source</th>
<th>Patients or species</th>
<th>Forward primer*</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Francis et al.</td>
<td>[347]</td>
<td>PBMCs††</td>
<td>Normal subjects</td>
<td>Within exon 10**</td>
<td>Within exon 10</td>
</tr>
<tr>
<td>Feliciello et al.</td>
<td>[350]</td>
<td>Retro-ocular tissue</td>
<td>Normal subjects</td>
<td>Exon 1 (residue 9)</td>
<td>Exon 10 (residue 1992)</td>
</tr>
<tr>
<td>Heufelder et al.</td>
<td>[344]</td>
<td>Cultured retro-ocular, pretibial, &amp; abdominal fibroblasts</td>
<td>TAO patients, &amp; normal subjects</td>
<td>Exon 1 (residue 109)</td>
<td>Exon 9 (residue 790)</td>
</tr>
<tr>
<td>Roselli et al.</td>
<td>[349]</td>
<td>All guinea-pig adipose tissues***</td>
<td>Guinea-pig</td>
<td>Exon 8 (residue 610)</td>
<td>Exon 10 (residue 1225)</td>
</tr>
<tr>
<td>Paschke et al.</td>
<td>[352]</td>
<td>EOM</td>
<td>Normal subjects</td>
<td>residue 57 and residue 999</td>
<td>residue 1239 residue 2301</td>
</tr>
<tr>
<td>Watson et al.</td>
<td>[360]</td>
<td>PBMCs††</td>
<td>GD§, GD+TAO, AI hypo† patients &amp; normal subjects</td>
<td>residues (1/24)</td>
<td>residues (282/259)</td>
</tr>
<tr>
<td>Wu et al.</td>
<td>[361]</td>
<td>Cultured GD+TAO+pretibial pretibial fibroblasts</td>
<td>GD+TAO+pretibial dermopathy patients &amp; normal subjects</td>
<td>residues (-11/8)</td>
<td>residues (754/773) (1265/1285)</td>
</tr>
</tbody>
</table>

††PBMCs: Peripheral blood mononuclear cells  * The nucleotide positions are numbered from the start codon

** This study was performed before the elucidation of the genomic organisation of the TSH-R

*** Demonstrated TSH-R transcripts in virtually all guinea-pig adipose tissues, with the exception of the Harderian gland of the orbit

§GD: Graves' disease  ‡TAD: thyroid-associated dermopathy  †AI hypo: autoimmune hypothyroidism
In 1992, Heufelder and Bahn suggested the presence of a functional TSH-R on cultured retro-ocular fibroblasts. This followed their demonstration of an enhanced expression of certain immunomodulatory molecules (i.e. intercellular adhesion molecule-1; ICAM-1) in vitro after binding by retro-ocular fibroblast monolayers, of IgGs derived from patients with TAO which had high TSH-R stimulating serum activity. Given the indirect evidence provided by these studies, the existence of a functional TSH-R on fibroblasts and other cell types residing in the retro-ocular fat/connective tissue remained speculative at this stage.

In 1993, using PCR and primers located in exons 1 and 10 of the TSH-R cDNA (see Table 1.7), Feliciello and colleagues reported amplification of a 2-kb transcript from whole retro-ocular tissue, but not from fibroblasts, lymphocytes, EOM or skeletal muscle inferring that adipocytes might also be a source of TSH-R. Also in 1993, Heufelder et al. detected by PCR and subsequent sequencing of overlapping amplification products, RNA encoding the extracellular domain of the human TSH-R in cultured orbital, abdominal skin, and pretibial skin fibroblasts from patients with TAO and from normal subjects. However, the same year using Northern blotting of mRNA derived from EOMs, and PCR amplification of an EOM cDNA library, Paschke et al. failed to detect transcripts corresponding to the extracellular portion of the human TSH-R. In 1994, Chang et al. reported, in fibroblasts derived from pretibial myxoedema, the presence of RNA encoding the extracellular domain of the TSH-R, but not the full length TSH-R. Another study reported the detection of human TSH-R gene expression in retro-ocular fibroblasts, using primers encompassing a portion (nucleotides 989-1235) of the extracellular domain of the human TSH-R. Endo et al. also used PCR to successfully amplify
cDNA encoding portions of the TSH-R from retro-ocular adipose tissue, EOM, and myxoedematous skin of patients with Graves' disease[348].

Recent communications by numerous investigators, claiming the detection of TSH-R RNA in multiple extrathyroidal tissues, have since only added to the confusion and controversy regarding the existence of TSH-R outside of the thyroid gland[354,365,366]. To add to the complexity, splice variants of variable lengths have been reported for the TSH receptor[197,367]. Also, Bahn's group demonstrated a genomic point mutation (an A for C substitution in the first position of codon 52) in the extracellular domain of the TSH-R in 2 among 22 patients with TAO studied, and considered that the mutant form of the receptor may have unique immunogenic properties[368]. However, Watson et al. reported that this coding region polymorphism is not associated with the occurrence of Graves' disease or TAO, investigating a much larger patient population[360]. In addition, Wu et al. have not been able to demonstrate any association between the coding region polymorphism, reported by Bahn et al., and TAO in patients who also had pretibial myxoedema[361].

Taken together, the above data suggest that TAO does not appear to be related to the variation of the TSH-R genetic sequences.

Furthermore, the finding of mRNA for the TSH receptor in fibroblasts using PCR does not guarantee the presence of functional or immunologically active TSH-R protein in these cells. Most RT-PCR studies attempting to detect TSH-R mRNA have used more than 30 PCR cycles, which means that any mRNA is at very low amounts.

In addition, a positive result obtained after many cycles could be due merely to illegitimate transcription and therefore of no pathophysiological relevance. Therefore, to support results obtained by PCR, demonstration of TSH-R gene
expression in retro-ocular tissues by alternative techniques such as Northern blotting, ribonuclease protection assay, and *in situ* hybridisation is desirable before concluding that TSH-R is expressed in orbital tissue.

Using a polyclonal Ab directed against a human TSH-R peptide (amino acids 352-367), Burch *et al.* detected immunofluorescence staining in retro-ocular and pretibial fibroblasts suggesting that it was specific for human TSH-R (\(-\)like) protein \(^{369}\). To verify these data, Heufelder performed immunofluorescence staining of retro-ocular and pretibial fibroblasts derived from 2 patients with TAO and pretibial dermopathy, using an affinity-purified polyclonal antibody directed against human TSH-R extracellular domain \(^{370}\). Specific, punctate immunoreactivity was detected in the cytoplasm and perinuclear area of methanol-fixed retro-ocular and pretibial fibroblasts derived from both patients, and cell-membrane associated immunoreactivity was detected in these cells postfixed with paraformaldehyde \(^{370}\). In contrast, no immunoreactivity was detected in abdominal fibroblasts derived from these same 2 individuals, in dermal fibroblasts from 2 patients with systemic scleroderma, and in various cell lines \(^{370}\).

More recently, by high stringency RT-PCR, Southern analysis, and direct sequencing of PCR products, the presence of both full length and splice variant TSH-R mRNA has been identified in EOM but not non-ocular skeletal muscle samples from normal individuals \(^{371}\). In addition, increased expression of both intact (2.4 \(\text{kb}\)) and variant (1.3 \(\text{kb}\)) TSH-R mRNA and of TSH-R protein was detected in retro-ocular fibroblasts *in vitro* following 72 hour treatment with TSH, suggesting that the expression of this receptor in the orbit *in vivo* may be stimulated by TSH or other TSH-R ligands \(^{372}\). Lately, work from two groups has directed attention towards
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retro-ocular fat/connective tissue and in particular retro-ocular pre-adipocytes as the source of TSH-R expression in the orbit. TSH-R transcripts of 4.8, 1.8, and 1.6 kb were visible in Northern blots performed with RNA from a sample of orbital fat from a patient with TAO and were as clearly visible, as in the thyroid but not in normal tissue \[^{373,374}\]. Therefore, Ludgate's group hypothesised that in TAO, TSH-R transcripts are elevated probably due to an increased number of cells, in particular of pre-adipocytes in orbital adipose tissue. Also, Heufelder's group showed that TSH-R is demonstrable in early passage pre-adipocyte retro-ocular fibroblast cultures that contain a subpopulation of adipocytes. Subsequent passaging of these cells resulted in the loss of both TSH-R expression and adipocyte staining, suggesting that both the expression of this receptor and the accumulation of adipose tissue in the orbit in TAO may be induced \textit{in vivo} perhaps by humoral factor(s) not present in the cell culture environment \[^{375}\]. Ludgate's group have hypothesised that in TAO, TSH-R transcripts are elevated due to an increased number of pre-adipocytes in retro-ocular adipose tissue in addition to increased expression.

Further studies are required to confirm these results and to determine whether TSH-R expressed by cells residing in the orbit is functionally active and/or immunologically relevant for the pathogenesis of TAO, since cross-reactivity with TSH-R expression in the thyroid represents an attractive explanation for the association of TAO with Graves' hyperthyroidism.

1.5.4 Adhesion molecule expression in TAO

Adhesion molecules play an important role in lymphocyte activation and localisation, antigen presentation, T cell co-stimulation, as well as various effector/target cell functions at the sites of inflammatory or immune...
processes\(^{[153,376]}\). In 1992 we demonstrated, by immunohistochemistry, that ICAM-1, E-selectin, vascular cell adhesion molecule-1 (VCAM-1) as well as their counterpart molecules CD11a, CD11b, CD11c were overexpressed on vascular endothelial cells and leukocytes of frozen sections of EOM biopsies from patients with active TAO \(^{[377]}\). The same year, Heufelder and Bahn reported that IFN-γ, TNF-α, and IL1-α strongly enhanced surface expression of ICAM-1 in both normal and TAO retro-ocular fibroblasts \textit{in vitro} \(^{[363]}\). The same group also showed strong immunoreactivity for ICAM-1 on frozen sections of retro-ocular fat/connective tissue specimens and EOM tissue from one patient \(^{[291]}\). Furthermore, in addition to hyaluronic acid receptor (CD44) \(^{[378]}\), VCAM-1 expressed by endothelial cells and retro-ocular fibroblasts, as well as VLA-4 expressed by lymphocytes were found to participate in the interactions between retro-ocular fibroblasts and lymphocytes \(^{[379,380]}\). In addition, Heufelder and Bahn reported the detection of an increased concentration of functionally active soluble ICAM-1 (sICAM-1) in sera of patients with clinically active, severe TAO \(^{[381]}\). The changes in sICAM-1 serum levels appeared to correlate with the degree of periorbital inflammatory activity in TAO and the response to anti-inflammatory therapy \(^{[381]}\). Recently, circulating sICAM-1 levels of euthyroid TAO patients were also reported to correlate with the response to octreotide treatment \(^{[267]}\).

Although the origin of sICAM-1 in sera of patients with TAO is not known, activated vascular endothelial cells, fibroblasts, and mononuclear cells within the inflamed TAO tissues, are potential sources.

In summary, there is evidence of increased expression of adhesion molecules and their appropriate counter-receptors on mononuclear cells, endothelial cells, and fibroblasts in retro-ocular fat/connective tissue TAO specimens, which is likely to be
due to cytokines secreted by the inflammatory infiltrate present in these tissues. However, their expression has not been examined in detail in sufficient EOM specimens for the various stages of TAO.

### 1.5.5 Role of the EOM fibres in TAO - bystander or target?

A dichotomy of opinion still exists about what is the primary target cell in TAO; the fibroblast or the EOM fibre. However, reports on the histopathology and immunohistochemistry of EOMs have shown no evidence of muscle cell destruction or autoimmune inflammation markers adjacent to the inflammatory infiltrate. Recently, an immunohistochemical study demonstrated HLA class II expression on affected EOM fibres, albeit only in 4 of the 38 patients studied, although 3 other studies have not been able to confirm these findings. Overall, the bulk of histologic and experimental evidence points to orbital fibroblast as the primary autoimmune target/effector cell in TAO.

### 1.5.6 Role of T cells in TAO

T cells have been proposed to play a central role in the pathogenesis of TAO, not least because organ-specific B cell autoimmune responses are generally T cell-dependent.

**(6a) T cell subsets**

T cell phenotype studies on circulating T cells in Graves' disease patients, in their majority, did not indicate any additional associations with the presence of TAO. Immunohistochemical studies of the EOM-infiltrating lymphocyte subsets in TAO reported predominance of T cells which comprised CD4+ and CD8+ cells in equal numbers and were mainly CD45RO+. In contrast, immunohistochemical
characterisation of the T cell subsets on retro-ocular fat/connective tissue from patients with severe, active TAO showed a predominance of CD8\(^+\) T lymphocytes\(^{[291]}\).

\textit{(6b) T cell receptor (TCR) variable (V) region usage}

The majority of retro-ocular T cells belong to the \(\alpha/\beta\) phenotype \(^{[292]}\). Molecular analysis of the retro-ocular T cell antigen receptor repertoire in untreated patients with severe, active TAO of recent onset has been reported in only one study. This indicated a marked degree of restriction of T cell receptor variable region gene families, suggesting that these T cells represent a primary, oligoclonal immune response directed against a limited number of antigenic epitopes within the retroorbital space \(^{[292]}\). However, studies in thyroid gland tissue have been contradictory \(^{[84,137-143]}\).

\textit{(6c) Cytokine production and expression}

Between 1972 and 1980, cytokines produced by Peripheral Blood Mononuclear Cells (PBMCs) sensitized to specific antigens derived from crude orbital, thyroid as well as lacrimal tissue extracts and membrane fractions were measured by their effects on mononuclear cell proliferation, migration, and adherence \(^{[386-389]}\). In 1989, Van der Gaag et al. reported the detection of a circulating factor with migration inhibition factor activity for monocytes in 67\% of patients with active TAO compared to 11\% of those with inactive disease \(^{[390]}\). Using the leukocyte procoagulant activity assay -which is based on the cellular collaboration between lymphocytes and monocytes- specific reactivity against soluble EOM and orbital connective tissue membrane fractions in TAO was described in 1992 \(^{[391]}\). In addition, in 1993 de Carli et al. demonstrated that retro-ocular T cell clones from TAO patients (\textit{produced in IL-2-conditioned medium and stimulated with}
phytohaemagglutinin (PHA)), with a balanced expression of CD4^+ and CD8^+ phenotypes, had a Th1-like cytokine profile (secreting IL-2, IFN-γ, and TNF-α but not IL-4 or IL-5) and were able to display non-specific cytotoxicity\textsuperscript{[392]}. In 1994, Grubeck-Loebenstein \emph{et al.} reported that retro-ocular T cell lines from TAO patients \emph{(cultured in IL-2 and further stimulated with anti-CD3 antibody)} were CD8^+CD45RO^+ and secreted IL-4, IFN-γ and IL-10 upon activation\textsuperscript{[393]}. The use of anti-CD3 antibody in the second study rather than PHA may have helped to maintain the CD8^+ content of the infiltrating T cells in culture\textsuperscript{[394]}.

Studies of in vivo cytokine expression in ocular tissues from TAO patients have been limited. Heufelder and Bahn have detected by immunohistochemistry IFN-γ, IL1-α and TNF-α in retro-ocular fat/connective tissue specimens from TAO patients but not normal controls\textsuperscript{[395]}. The more sensitive RT-PCR has shown both IL-4 and IL-10 expression in 2 of 5 retro-ocular fat specimens and in a single EOM biopsy obtained from TAO patients, but not IFN-γ in any of the samples analysed, indicating a Th2-like immune response\textsuperscript{[396]}. The observed discrepancies between the in vivo and in vitro cytokine data could probably be attributed to the culture conditions, where multiple agents are used to stimulate cell growth.

\textbf{(6d) Role of cytokines in TAO}

Cytokine effects on retro-ocular fibroblasts have been studied in vitro. In 1994, Heufelder and Bahn reported that retro-ocular fibroblast proliferation is stimulated by cytokines, including IL-1α, IL-4 and TGF-β, but not IL-2 or IL-6\textsuperscript{[397]}. Fibroblast function has also been shown to be modulated by cytokines. IFN-γ, TNF-α, TGF-β and IL-1 can induce GAG production, whereas IFN-α and IL-6 have no effect\textsuperscript{[398-401]}. Fibroblast stimulation by these cytokines is further enhanced by
hypoxia, which may explain the adverse effects of smoking in TAO\textsuperscript{[400]}. In addition, cytokines stimulate fibroblasts to synthesise collagen and connective tissue\textsuperscript{[402]}. Furthermore, they may also indirectly affect the inflammatory process through augmenting adhesion molecule (see above), MHC class II and heat shock protein (HSP) expression in the retro-ocular fat/connective tissue\textsuperscript{[63,210]}.

\textbf{(6e) Antigenic reactivity}

When Grubeck-Loebenstein \textit{et al.} screened the retro-ocular T cell lines they developed for their antigenic reactivity, all six lines proliferated in response to stimulation with autologous retro-ocular fibroblasts in an HLA class I-restricted manner, but did not recognise autologous PBMCs, crude EOM extract, allogeneic cells, or purified protein derivate of \textit{Mycobacterium tuberculosis}\textsuperscript{[393]} in contrast to PBMC-derived T cell lines. More recently, Otto \textit{et al.} tested the antigenic reactivity of 17 retro-ocular fat/connective tissue-derived T cell lines from 6 patients with TAO\textsuperscript{[403]}. All 17 T cell lines responded significantly to autologous adipose/connective tissue protein fractions 6-10 kDa and 19-26 kDa, but not to tuberculin\textsuperscript{[403]}, suggesting that the 19-26 kDa fraction may be identical to the reported 23 kDa fibroblast protein\textsuperscript{[404]}. Incubation of PBMCs with autologous orbital protein fractions gave similar results. However, positive responses were not found to abdominal adipose or muscle proteins which were used as controls. Phenotypic analysis of 10 of the 17 orbital T cell lines indicated that 6 lines consisted predominantly of CD4\textsuperscript{+} cells in accordance with previous findings reported from the same group when phenotypical screening of 62 orbital T cell clones revealed a CD4\textsuperscript{+}/CD8\textsuperscript{+} ratio of 8.2, contrasting with a normal ratio of 2.1 in PBMCs\textsuperscript{[405]}.

The above data support the view that retro-ocular fibroblasts represent a major T cell target in TAO. However, it should be taken into consideration that they
are based on *in vitro* experiments and might be subject to influences arising from the experimental conditions.

### 1.5.7 Heat shock protein expression in TAO

Heat shock proteins (HSPs) have been detected, both *in vitro* and *in vivo* in fibroblasts from specimens of TAO patients but not retro-ocular tissue from normal controls \([406-408]\). Various cytokines, including IFN-γ and TNF-α have been shown to enhance HSP expression in cultured retro-ocular fibroblasts from TAO patients but not normal controls \([406]\). In addition, IL-1, IL-6 and TGF-β have been shown to increase HSP expression (72 kDa) in fibroblasts from both normal and TAO retro-ocular tissue \([406]\). In contrast, IL-2, granulocyte macrophage-colony stimulating factor (GM-CSF) and EGF had no effect on HSP expression in cultured retro-ocular fibroblasts \([406]\). It is thus likely that interaction of various immunomodulatory proteins, including HLA-DR, adhesion molecules, HSPs, plays a part in antigen presentation and T cell recognition of antigenic epitopes in orbital tissue in TAO.

### 1.5.8 The role of IGF-1 in TAO

Insulin-like growth factor 1 (IGF-1) is an important paracrine and autocrine growth factor with widespread effects on connective tissue, muscle and adipose tissue \([409]\). In adult life circulating IGF-1 originates predominantly from liver, where its synthesis is under regulation by pituitary growth hormone (GH) \([409]\). Several binding proteins limit the bioavailability of IGF-1 which is regulated by other hormonal influences. IGF-1 is also produced locally by a variety of tissues including connective tissue cells, and is thought to act in a paracrine and autocrine fashion \([410]\). IGF-1 has been shown to stimulate fibroblast proliferation and production of
collagen and GAGs\cite{411}. Therefore, IGF-1 has been implicated in the pathogenesis of TAO, although the evidence for its role is weak. Hypophysectomy (with consequent reduction in pituitary GH secretion and plasma IGF-1 levels) was used several years ago in the treatment of severe TAO, apparently with some success\cite{412}. More recently, the somatostatin receptor analogue, octreotide (which inhibits GH secretion and causes a reduction in circulating IGF-1 concentrations) was used with success in the treatment of TAO and pretibial myxoedema\cite{265}. Further evidence came from the demonstration of increased “IGF-1 immunoreactivity” in orbital tissues from a patient with TAO\cite{413}. IGF-1 binding sites on cultured orbital fibroblasts were demonstrated\cite{414}. Interestingly the density of binding sites was shown to be significantly higher in orbital than dermal fibroblasts\cite{414}.

The mechanism(s) by which IGF-1 may be involved in TAO are unknown, but include direct stimulation of IGF-1 receptors on orbital fibroblasts by specific autoantibodies. Some evidence for this hypothesis comes from binding experiments using IgG from patients with TAO\cite{414}. The localisation of the disease process to the orbit(s) would be difficult to explain given the almost ubiquitous distribution of IGF-1 receptors. Alternatively, local production of IGF-1 by orbital tissues may be driven by the inflammatory response in the orbit; the autocrine/paracrine effects of high local IGF-1 levels would further stimulate GAG and collagen production.

\textbf{1.5.9 Role of retro-ocular fibroblasts and glycosaminoglycans in TAO}

Histologic studies in TAO have shown that the affected tissues become infiltrated with activated T cells\cite{288,290,298} and accumulate increased amounts of glycosaminoglycans\cite{288,290,294-296,415}, in particular hyaluronan. Fibroblasts within the
connective tissue of the EOMs and the orbital fat compartment are a major source of these GAGs, and thus are thought to play a primary role in the development of TAO. Phenotypically, orbital fibroblasts display differences compared to dermal fibroblasts. They express Thy-1, a 25 kDa surface glycoprotein associated with cell signalling, in a distinct bimodal pattern as compared to the uniform distribution of Thy-1 in dermal fibroblasts. Orbital fibroblasts in culture exhibit shapes distinct from those of dermal fibroblasts despite having similar ultrastructural features. Thy-1-positive lung fibroblasts are more spindle-shaped and contain more intracellular lipid than fibroblasts not expressing Thy-1. In contrast, orbital fibroblasts in parent cultures expressing Thy-1 do not differ morphologically from those without the marker, despite the substantial shape heterogeneity that exists within each culture strain. However, clones of orbital fibroblasts derived from a common parent culture take on distinct morphologies that are consistently maintained despite serial passage and substantial time in culture. This observation suggests that it may be possible in future, upon further examination, to identify biochemical markers that unify a particular subpopulation of similarly appearing orbital fibroblasts.

Furthermore, functional specialisation of orbital fibroblasts has been reported. Regarding hyaluronan synthesis, orbital fibroblasts are considerably more susceptible to the up-regulating effects of IFN-γ and leukoregulin (a 50 kDa T cell-derived cytokine, influencing the synthesis of collagenase, stromelysin-1, collagen and hyaluronan) than are dermal fibroblast cultures. Moreover, plasminogen activator inhibitor type 1 (PAI-1), a serine protease inhibitor that exerts substantial influence on the rate of turn-over of the extracellular matrix, is differentially
expressed and regulated in orbital fibroblasts [424-427]. Based on the characteristic profile expression of receptors, gangliosides, and patterns of protein induction [424,425,427-429], Smith and colleagues have proposed that distinct subpopulations of orbital fibroblasts exist, at least in culture [428]. The same group, subsequently hypothesised that a subset of fibroblasts may be responsive to adipogenic signals, and recently reported that cultured orbital fibroblasts, but not dermal or perimysial fibroblasts from EOMs, can differentiate into mature, lipid-filled adipocytes in response to appropriate differentiation stimuli [430]. They therefore suggested that these in vitro observations may represent the in vivo process that contributes to excess orbital adipose tissue volume in TAO.

Finally, orbital fibroblasts have been shown to be more sensitive to the induction of HLA-DR after treatment with interferon-γ than abdominal skin fibroblasts [402], implying that they may play a role in antigen presentation and perpetuation of the autoimmune response in TAO. Messenger RNA transcripts encoding the human TSH-R have been detected in orbital fibroblasts [370], enhancing further the potential role of the retro-ocular fibroblast as a target as well as an effector cell in TAO.
### 1.6 PATHOGENESIS OF TAO - A WORKING HYPOTHESIS

Based on our current state of knowledge, the leading hypothesis on the pathogenesis of TAO proposes that autoreactive T cells that have escaped deletion are directed against antigens (as such, or altered) shared by the thyroid gland and the orbital tissue (EOMs, retro-ocular fat/connective tissue). These T cells are recruited by circulating or cell-surface adhesion molecules (which may also play a co-stimulatory role in T cell activation and facilitate antigen recognition) and thereby infiltrate the perimysium of the extraocular muscles and the retro-ocular tissue. Once infiltrated, activated T cells (and macrophages), after antigen recognition, secrete cytokines into the surrounding tissue. In consequence of the action of these cytokines, heat shock proteins 70 and 72, intercellular adhesion molecules, and HLA-DR are expressed on orbital fibroblasts (including those fibroblasts present in the perimysium of EOMs, which do not appear to be immunologically different from fibroblasts located in the retro-ocular connective tissue), fomenting thus the autoimmune response in the orbital connective tissue. Cytokines and growth factors released both from infiltrating inflammatory and residential cells act upon orbital fibroblasts in a paracrine and autocrine manner to stimulate further the expression of immunomodulatory molecules, fibroblast proliferation and glycosaminoglycan overproduction. GAGs, in view of their hydrophilic nature, are ultimately chiefly responsible for the clinical manifestations of the disease, including periorbital oedema, increased extraocular muscle volume, and proptosis. Despite the fact that this model bears several unanswered questions, it is clear that cytokines are widely involved in both triggering and perpetuating the cascade of reactions occurring in the EOM and retro-ocular fat/connective tissue of TAO patients which eventually lead to
clinical disease.

Whether ocular immune reactions in TAO patients are predominantly humoral or cell-mediated remains still uncertain, although it is highly likely that both are important for full clinical expression and propagation of the autoimmune process within the orbit.
1.7 OBJECTIVES OF THIS THESIS

The aims of the studies undertaken for this thesis were:

(a) To determine whether vascular and cellular adhesion molecules are expressed on affected EOMs from patients with TAO, and if so whether there are any differences in the pattern and degree of expression related to the different stages of activity of the disease? For this, biopsies of affected EOMs were collected from two groups of TAO patients. The first group consisted of patients with early, active TAO, and the second with late, inactive TAO. The pattern of adhesion molecules ICAM-1, E-Selectin, V-CAM as well as that of the leukocyte integrins CD11a/CD18, CD11b/CD18, and CD11c/CD18 was assessed by means of immunohistochemistry.

(b) To characterise the mononuclear cell infiltrate present in affected EOMs from TAO patients at different stages of disease, and to investigate the expression and localisation of HLA-DR during the different stages of disease activity. For this, immunohistochemistry was carried out using the relevant monoclonal antibodies and an avidin-biotin system on biopsies of affected EOMs collected from patients with early, active TAO, and from patients with late, inactive TAO.

(c) To determine the EOM infiltrating T cell phenotype, antigenic reactivity and cytokine profile in TAO patients. For this, biopsies of affected EOMs were taken and the infiltrating T cells isolated and expanded in vitro with mitogen. Their phenotype was determined by flow cytometric (FACS) analysis and compared to peripheral blood-derived T-cell lines, treated in the same way from the same patient. Cytokines present in the supernatant after mitogen stimulation of the T-cell lines were assayed by ELISA. In addition, cytokine mRNA present at the time of biopsy was determined by rapid RNA extraction from EOMs and reverse transcription-
amplification with specific cytokine oligonucleotide probes (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IFN-γ, TNF-α). In the T-cell lines from two patients, proliferation assays were carried out with antigens derived from thyroid gland, EOM and a TSH-receptor preparation.

(d) To determine the ultrastructural localisation of GAGs in the extraocular muscles of patients with TAO and to see if the quantity and type of GAGs present in blood and urine are markers of the disease. For this, biopsies of affected EOMs were taken and studied by transmission electron microscopy (TEM). These were either fixed conventionally for TEM, or in 0.5% tannic acid and others for immunogold staining. Serum hyaluronan (HA) was measured using a radioimmunoassay in patients with TAO as well as control subjects, and urinary GAG levels assessed by photometric quantitation of hexuronic acid after reaction with carbazole. The excretion pattern of the urinary GAGs was determined by discontinuous electrophoresis.
1.8 BACKGROUND TO THE CURRENT STUDY -
(A) ADHESION MOLECULES

In this report I have studied the expression of adhesion molecules in TAO. Adhesion molecules and adhesion receptors participate in orchestrating vital biological phenomena, such as embryogenesis, cell growth and differentiation, as well as wound repair\[431\]. They also play an important role in lymphocyte activation and localisation, antigen presentation, T cell co-stimulation, in addition to various effector/target cell functions at the sites of inflammatory or immune processes\[153,376\].

There are four major families of adhesion receptors expressed on cell surfaces: (a) the selectins, (b) the integrins, (c) the members of the immunoglobulin family (including chemoattractants), and (d) the cadherins. However, there is no evidence that cadherins are involved in the pathogenesis of TAO and thus they will not be considered further.

1.8.(A).1 The Selectins

The name selectin capitalises on the derivation of lectin and select from the same Latin root, meaning to separate by picking out. Selectins mediate functions unique to the vasculature, the tethering of flowing leukocytes to the vessel wall, and the formation of labile adhesions with the wall that permit leukocytes subsequently to roll in the direction of flow\[432\]. Selectins can mediate tethering of a flowing cell within a millisecond whereas other adhesion receptors require minutes to develop similar adhesive strength and do not mediate rolling\[432,433\]. Unlike most adhesion molecules, which bind to other proteins, the selectins interact with carbohydrate ligands on leukocytes and endothelial cells. The three members of the selectin family
(see Fig. 1.21) were named after the tissues in which they were first identified, and all three play a part in leukocyte rolling. L-selectin is constitutively expressed by most leukocytes and mediates the homing of lymphocytes to lymph nodes. P-selectin is stored in α-granules of platelets and Weibel-Palade bodies of endothelial cells, moving promptly to the plasma membrane on stimulation of these cells.

E-selectin, previously known as endothelial-leukocyte adhesion molecule-1 (ELAM-1), appears on endothelial cells after they have been activated by inflammatory cytokines; the small amount of E-selectin in many vascular beds appears to be important for the migration of leukocytes.
leukocytes. E-selectin supports adhesion of neutrophils, monocytes, eosinophils, and some lymphocytes. It is biosynthesized and expressed in response to bacterial endotoxin and the inflammatory cytokines interleukin 1 (IL-1) and tumour necrosis factor (TNF). E-selectin expression reaches maximal levels by 4-6 h and returns toward basal levels over 24-48 h\(^{[443]}\) (see Fig. 1.22).

![Kinetics of endothelial adhesion molecule expression](image)

**Fig. 1.22** Kinetics of endothelial adhesion molecule expression after exposure to IL-1, TNF, or endotoxin (E-selectin, VCAM-1, ICAM-1) or to thrombin, histamine, terminal complement components or H\(_2\)O\(_2\) (P-selectin). ICAM-1 is also inducible by interferon-\(\gamma\)\(^{[443]}\) (Reproduced with permission, from the Annual Review of Medicine, Volume 45, © 1994, by Annual Reviews Inc.)
All selectins appear to recognise a sialylated carbohydrate determinant on their counter-receptors \(^{[434,435,444]}\). E-selectin and P-selectin recognise carbohydrate structures that are distinct, but are both closely related to the tetrasaccharide sialyl Lewis \(\text{x}\) and its isomer sialyl Lewis \(\text{a}\) (PSGL-1, P-selectin glycoprotein ligand). The carbohydrate ligands for L- and P-selectin are linked to specific mucin-like molecules. L-selectin recognises at least 2 mucins in high endothelial venules (HEV): glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), which is secreted \(^{[445]}\), and CD34, which is on the cell surface \(^{[446]}\). The carbohydrate ligand for L-selectin is related to sialyl Lewis \(\text{a}\) and \(\text{x}\) \(^{[447,448]}\).

![Diagram of endothelial cell and leukocyte surface molecules](image)

**Fig. 1.23** Cell surface molecules involved in endothelial-leukocyte adhesion \(^{[443]}\). (Reproduced with permission, from the Annual Review of Medicine, Volume 45, © 1994, by Annual Reviews Inc)
Interactions of all selectins with their carbohydrate ligands appear to be essential for the initial contact of leukocytes with activated endothelium (see Fig. 1.23, page 106). In particular, it has been demonstrated that the tetrasaccharide sialyl Lewis x (sLe^\(^x\)) and related terminal sugars expressed by neutrophils, monocytes, and some lymphocytes can act as ligands for E-selectin\(^{[434,435]}\).

### 1.8(A).2 The Integrins

Integrins are perhaps the most versatile of the adhesion molecules. Their adhesiveness can be rapidly regulated by the cells on which they are expressed. They are membrane glycoproteins with two noncovalently associated subunits, designated \(\alpha\) and \(\beta\)\(^{[153,449]}\). Each subfamily is defined on the basis of a common \(\beta\) subunit; \(\beta_1\), \(\beta_2\), \(\beta_3\) defining the VLA (very late activation) antigens, the leukocyte integrins and the cytoadhesins respectively. Of all integrins, five are important in the interaction of leukocytes with endothelial cells: the three members of the leukocyte integrin subfamily (LFA-1, Mac-1, and p150,95), and the two members of the \(\alpha^4\) integrin subfamily (VLA-4 and LPAM-1) (see Fig. 1.24, next page).

The 3 \(\alpha\beta\) heterodimeric membrane glycoproteins of the leukocyte integrin family\(^{[449,450]}\) share a common \(\beta\) subunit (\(\beta_2\)), designated CD18. The \(\alpha\) subunit of each of the 3 members is designated CD11a for lymphocyte function associated antigen-1 (LFA-1), CD11b for macrophage antigen-1 (Mac-1) and CD11c for p150,95. Association of the \(\alpha\) and \(\beta\) subunit precursors occurs in the Golgi apparatus\(^{[451]}\) and the assembled receptors are then transported to the cell surface or the intracellular stores\(^{[452,453]}\).
The expression of integrins is restricted to leukocytes and haemopoietic precursor cells CD11a/CD18 (or LFA-1) is expressed on virtually all immune cells except for some tissue macrophages \(^{[454,455]}\). CD11b/CD18 (or Mac-1) is found on granulocytes, monocytes, macrophages, large granular lymphocytes and some immature CD5\(^+\) B-cells \(^{[456,457]}\). CD11c/CD18 (or p150,95) has a similar distribution to Mac-1, but also expressed on some cytotoxic T cells and activated lymphocytes, and is also a marker for hairy cell leukaemia \(^{[458,459]}\). The conversion from naive to memory T cells results in increased expression of CD11a, together with other “activation” molecules such as CD2 and LFA-3.

**Integrin Family**

![Integrin Family Diagram](Image)

**Fig. 1.24**. Representative integrin family adhesion receptors \(^{[442]}\). Although schematic, the overall size and shape of the integrins is shown to scale, (bar: 10 nm). \(^{[443]}\) (Reprinted with permission from *Nature* \(^{[442]}\) Copyright (1990) Macmillan Magazines Limited, and the author’s permission).

There is a degree of overlap between the different binding partners used by the different integrins. CD11a/CD18 binds to ICAM-1 at sites in its N-terminal Ig domains (see Fig. 1.23, page 106). CD11b/CD18, whose expression is upregulated on granulocytes and monocytes after various stimuli, has a variety of ligands.
including ICAM-1 (binding in its third Ig domain). CD11a or CD11b require activation before they are able to bind to ICAM-1. This activation of surface-resident integrins can be initiated by stimuli including complement components (e.g. C5α), cytokines (e.g. IL-4, IL-8, IFN-γ), and lipid mediators such as PAF (platelet activating factor). For instance, IFN-γ-treated murine macrophages and IL-4-treated B-cell lymphoma cell lines increase their expression of CD11a. The existence of integrins in active and inactive states is a characteristic of this class of adhesion molecules.

### 1.8.(A).3 The members of the Ig superfamily

Adhesion molecules members of the immunoglobulin superfamily (Ig-CAMs), expressed on endothelium, represent the binding proteins for integrins expressed on leukocytes (see Fig. 1.25). The most clearly associated with leukocyte adhesion and diapedesis are ICAM-1 (CD54), ICAM-2, VCAM-1, and PECAM-1 (CD31).

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**Fig. 1.25** Immunoglobulin superfamily including the adhesion receptors. Overall size and shape of the immunoglobulin domains are shown to scale (bar: 10nm), although schematised. (Reprinted with permission from *Nature* [442] Copyright (1990) Macmillan Magazines Limited, and the author’s permission).
ICAM-1 is a transmembrane glycoprotein containing five Ig domains found on hematopoietic and non-hematopoietic cells. On vascular endothelium, ICAM-1 is present at low levels under normal conditions and is dramatically upregulated by endotoxin, IL-1, and TNF. Increased expression of ICAM-1 after cytokine induction is detectable \textit{in vitro} and \textit{in vivo} after 4-6 hours, and is maximal by 9-24 hours \cite{465-467} (see Fig. 1.22, page 105). The up-regulation of ICAM-1 persists for several days in cultured endothelial cells, and abundant ICAM-1 expression on endothelium and other cell types is a common characteristic of inflammatory and immune responses \cite{153,461}. Endothelial ICAM-1 supports the adhesion and transmigration of leukocytes through an interaction with $\beta_2$ integrins, particularly CD11a/CD18 \cite{153,462-464} (see Fig. 1.23, page 106). In most inflammatory reactions, it is likely that binding of ICAM-1 to activated $\beta_2$ integrins occurs after interactions of selectins with their carbohydrate ligands \cite{434,435,437,462,468}. ICAM-2, another counter-receptor on the target cell(s) for CD11a/CD18, unlike ICAM-1 is well expressed basally on endothelial cells and its mRNA is not increased by inflammatory mediators \cite{153}.

Endothelial VCAM-1 (INCAM-110), another IgCAM, is upregulated by endotoxin, IL-1, and TNF over several days \cite{461} (see Fig. 1.22, page 105). VCAM-1 supports the adhesion of lymphocytes, monocytes, and eosinophils through an interaction with its counter-receptor, the $\alpha^4\beta_1$ integrin (CD49d/CD29, VLA4). Neutrophils do not bear $\alpha^4\beta_1$ and consequently do not bind to VCAM-1. Interestingly, the cytokine IL-4 acts on endothelium to up-regulate VCAM-1 but not E-selectin \cite{469}. Thus, IL-4 is a candidate for the regulation of disease processes characterised by a paucity of neutrophils and an abundance of mononuclear
leukocytes or eosinophils.

## 1.8.(A).4 The role of endothelium in inflammation

Normally quiescent, the endothelium maintains blood fluidity by inhibiting coagulation and resisting the adhesion of blood leukocytes. In response to infection and tissue injury, however, endothelial cells become activated and express molecules that promote the host response. Through the generation of vasoactive compounds (e.g. PGI₂), lipid-based activators of leukocytes (e.g. platelet activating factor, PAF), chemotactic cytokines (e.g. IL-8), and specific cell surface adhesion molecules, the endothelium orchestrates the movement of fluid and leukocytes that characterises inflammation \(^{[461,470]}\).

![Diagram](image)

**Fig. 1.26** Three sequential steps provide the traffic signals that regulate localization in the vasculature. \(^{[471]}\). (Reproduced with permission, from the Annual Review of Physiology, Volume 57, © 1995, by Annual Reviews Inc.).
1.8.(A).5 Summary of the role of adhesion molecules in inflammation

Lymphocytes continually patrol the body for foreign antigen by recirculating from blood, through tissue, into lymph, and back to blood. Lymphocytes acquire a predilection, based on the environment in which they first encounter foreign antigen, to home or to recirculate through that same environment \(^{[472,473]}\). A lymphocyte may emigrate and recirculate many thousands of times during its life history. Recirculation of lymphocytes correlates with their role as antigen receptor-bearing surveillance cells \(^{[471]}\). They function as the reservoir of immunological memory, and recirculate through tissues to provide systemic memory. Granulocytes and monocytes do not recirculate. They emigrate from the bloodstream in response to molecular changes on the surface of blood vessels that signal injury or infection but do not return in viable form to the circulatory pool. The nature of the inflammatory stimulus and the mixture of chemoattractants and cytokines produced at inflammatory sites in vivo determine whether lymphocytes, monocytes, neutrophils, or eosinophils predominate, and thus exercise specificity in the molecular signals or "area codes" that are displayed on endothelium and control traffic of particular leukocyte classes.

These "traffic signal" or "area code" molecules are coexpressed on endothelium but act on leukocytes in a sequence during lymphocyte homing (see Fig. 1.26, page 111). Selectins (Step 1) allow cells to tether and roll, chemoattractants (Step 2) activate integrin adhesiveness, and the Ig family members (Step 3) bind integrins, resulting in cell adhesion to the vessel wall before diapedesis \(^{[471]}\).
1.8 BACKGROUND TO THE CURRENT STUDY -
(B) CYTOKINES & T CELLS

In this report I have also studied the cytokine production of extraocular muscle-derived T cell lines from patients with TAO and in collaboration with Professor A.P. Weetman and Dr. Ramzi Ajjan the pattern of cytokine gene expression in TAO and control EOMs ex vivo.

Cytokines are proteins or glycoproteins, which act as local messengers produced by cells of any type, but most frequently by leukocytes\[474\]. These proteins are essential components of most important biological processes, including cell growth, development, repair, fibrosis, immunity and inflammation. In most cases they are produced transiently after stimulation and in small quantities, unlike hormones which are produced constitutively. The term “cytokines” currently encompasses: (a) the interleukins, (b) the interferons (α, β, γ, ω), (c) the growth factors, (d) the tumour necrosis factors, and (e) the chemokines (chemotactic cytokines)\[474\].

With the exception of IL-6 which is frequently found in the serum in a bioactive form, and the active form of macrophage colony stimulating factor (M-CSF), most cytokines are not found in bioactive forms in plasma or serum. Typically, they act as signalling molecules, working at low concentrations, usually $10^{-10}$ to $10^{-13}$ mol/litre, or approximately 1 ng to 1 pg/ml. Their potency is due to the high affinity of their receptors, in the range of $10^{-9}$ to $10^{-12}$ mol/litre, and the low receptor occupancy needed for cell activation\[474\]. Every cell type expresses multiple cytokine receptors, with some receptors being virtually ubiquitous (i.e., IL-1, IL-6, TNF, IFN). However, the effects of a single cytokine on different cell types varies,
and this property is known as "pleiotropy" [475]. Another property of cytokines is that of overlapping biological effects, known as cytokine "redundancy" [475]. A common property of cytokines is that they can induce the production of other cytokines [476] and act in networks either as signalling molecules, in concert with other signals (i.e., via the T cell receptor for antigen) or in networks unrelated to antigen presentation such as that of TNF-α seen in inflammation [477].

Through their activities, cytokines may throw the normal immune response "off balance" and cause profound changes which may lead to autoimmunity. Likewise, many of the clinical and laboratory manifestations of autoimmune diseases may be attributed to the effects of cytokines.
**Table 1.8 Main sources and functions of the cytokines examined in this study**

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>SOURCE</th>
<th>MAJOR FUNCTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 (α and β)</td>
<td>monocytes, macrophages, endothelium, epithelium (including TFCs), neuronal &amp; glial cells, fibroblasts, keratinocytes</td>
<td>-induces inflammatory and immune responses (prostaglandin production, metalloproteinases etc.)&lt;br&gt;-upregulates adhesion molecules: (ICAM-1, VCAM-1, E-selectin)&lt;br&gt;-upregulates other cytokines: (IL-2, IFN, TNF, IL-6, IL-8 etc.)&lt;br&gt;-affects CNS and endocrine system (stimulates Hypothalamic Pituitary-Adrenal axis)</td>
</tr>
<tr>
<td>IL-2</td>
<td>T cells (exclusively)</td>
<td>-stimulates T cell proliferation and differentiation&lt;br&gt;-B-cell proliferation/antibody production&lt;br&gt;-enhances NK activity and production of LAK cells</td>
</tr>
<tr>
<td>IL-4</td>
<td>T&amp;B lymphocytes, basophils, mast cells, macrophages</td>
<td>-induces differentiation into Th2&lt;br&gt;-suppresses Th1 responses&lt;br&gt;-induces B-cell proliferation, differentiation, and Ig secretion (IgE and IgG1 especially)&lt;br&gt;-upregulates class II on B cells&lt;br&gt;-inhibits macrophage proinflammatory cytokine synthesis (IL-1, TNF, IL-6, etc)</td>
</tr>
<tr>
<td>IL-6</td>
<td>T&amp;B lymphocytes, monocytes, Mφs, hepatocytes, neurones, fibroblasts, endothelial cells, TFCs</td>
<td>-activation of haematopoietic cells&lt;br&gt;-stimulates T&amp;B proliferation and differentiation&lt;br&gt;-induces acute phase protein response, IFN, NK cell activation, platelet production, non-inflammatory mesangial cell proliferation&lt;br&gt;-activates osteolysis</td>
</tr>
<tr>
<td>IL-8</td>
<td>T cells, monocytes, neutrophils, endothelium, epithelium (including TFCs), fibroblasts, keratinocytes, hepatocytes</td>
<td>-chemotactic for neutrophils, lymphocytes and basophils&lt;br&gt;-enhances neutrophil adhesion to endothelial cells and keratinocyte proliferation</td>
</tr>
</tbody>
</table>
### Table 1.8 (continued). Main sources and functions of the cytokines examined in this study[^475,478-491]

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>SOURCE</th>
<th>MAJOR FUNCTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>T&amp;B cells, Mφs, keratinocytes</td>
<td>- suppresses Mφ cytokine production, - inhibits antigen presentation, - down-regulates cytokine receptor expression, - strong B cell activation, Ig production</td>
</tr>
<tr>
<td>IL-13</td>
<td>T cells TFCs</td>
<td>- B cell co-activation, Ig secretion (IgG, and IgE especially), - up-regulates class II on B cells - activator of most cells &amp; basophils - suppresses Mφ pro-inflammatory cytokine production</td>
</tr>
<tr>
<td>INF-γ</td>
<td>T cell NK cells</td>
<td>- induces class II &amp; class I - strongest activator of monocytes, augmenting cell surface receptors &amp; priming for cytokine production, - antiviral activity, - blocks Th2 activity (IL-4 &amp; IL-5 production), promotes DTH, - inhibits B cell proliferation &amp; IgE production - activates neutrophils and NK cells</td>
</tr>
<tr>
<td>TNF-α</td>
<td>neutrophils NK/LAK cells endothelial cells smooth muscle cells</td>
<td>- upregulates other cytokines (IL-1, IFN, IL-6, IL-8 etc.) - upregulates adhesion molecules - upregulates acute phase proteins - upregulates T and B cell activation - induces inflammation, e.g. prostaglandins, leukotrienes - suppresses the bone marrow - tumour cell cytotoxicity - endogenous pyrogen</td>
</tr>
</tbody>
</table>

Mφs: Macrophages; NK cells: Natural killer cells; TFCs: Thyroid follicular cells
DTH: Delayed type hypersensitivity; LAK cells: Lymphokine activated killer cells
Pro-inflammatory cytokines: IL-1α, IL-1β, IL-2, IL-6, TNF-α, IFN-γ, lymphotoxin
Anti-inflammatory cytokines: IL-4, IL-10, IL-13, TGF-β
1.8.(B).1 Cytokines & T cell subsets

The definitive T cell marker is the T cell receptor (TCR), which consists of either αβ or γδ polypeptide chains \(^{492,493}\). The other molecule associated with the TCR is CD3. The majority of T cells (95%) use the αβ heterodimer in antigen recognition. TCR αβ bearing cells can be subdivided into two distinct non-overlapping populations: the T helper (Th) subset which is CD4\(^+\) and the cytotoxic T lymphocyte subset (CTL) which is CD8\(^+\). CD4\(^+\) helper cells recognise the antigen presented by MHC Class II molecules whereas the CD8\(^+\) cytotoxic T cells recognise the antigen presented by Class I molecules. However, there are also examples of CD4\(^+\) Class II restricted CTLs \(^{494}\).

Naive CD4\(^+\) T cells when stimulated produce IL-2 as their major lymphokine \(^{495}\). Upon priming, these cells develop into cells that produce either IFN-γ, TNF-β, and IL-2; or IL-4 and its congeners. A cellular basis for this distinctive pattern of lymphokine production was provided by the seminal observation of Mosmann & Coffman \(^{496}\) that long term clones of mouse CD4\(^+\) T cells could be subdivided into those that produce IL-2, IFN-γ, and TNF-β (Th1 clones) and those that produce IL-4, IL-5, IL-6, IL-10, and IL-13 (Th2 clones). The separation of T cells into Th1 and Th2 categories according to their function in cell mediated or humoral immunity is a concept that is proving useful in understanding the immune system \(^{496}\).

Although the Th1 and Th2 subsets are relatively clearly defined in the murine immune system, these categories are not so clear-cut in the human immune system where the designations Th1-like and Th2-like have been suggested. However, there is a large body of evidence suggesting the existence of polarised human T cell
mediated effector responses, reminiscent of Th1 and Th2 subsets described for mouse
T cells, and very recently a surface marker (the IL-12 receptor β2 subunit) for human
Th1 cells has been reported\textsuperscript{[497]}. Human Th1-like cells preferentially develop during
infections by intracellular bacteria\textsuperscript{[498-501]}, protozoa\textsuperscript{[502]}, and viruses\textsuperscript{[503]}, whereas
Th2-like cells predominate during helminthic infestations\textsuperscript{[504-506]} and in response to
common environmental allergens in patients with contact hypersensitivity or atopic
allergy\textsuperscript{[507]}. Strongly polarised human Th1-type and Th2-type responses can also
promote different immunopathological reactions. Th1 cells are responsible for both
humoral and cell-mediated immune responses: antibody production of the IgG2a
class (in mouse), macrophage activation, antibody-dependent cell cytotoxicity and
delayed type hypersensitivity (DTH). Th2 cells provide optimal help for humoral
immune responses. These include IgE and IgG1 (in mouse) or IgG4 (in man) isotype
switching, and mucosal immunity, through production of mast cell and eosinophil
growth and differentiation, as well as facilitation of IgA synthesis\textsuperscript{[496,508]}. In the
absence of clear polarising signals, CD4\textsuperscript{+} T cell subsets with a less differentiated
lymphokine profile than Th1 or Th2 cells, designated Th0, usually arise, and these
mediate intermediate effects depending upon the ratio of lymphokines produced and
the nature of the responding cells\textsuperscript{[509-512]}. Th0 cells are considered to be obligatory
precursors of Th1 and Th2 cells (Fig. 1.27, page 119).
Several factors, including the dose of antigen, the type of antigen-presenting cell (APC), the major histocompatibility complex (MHC) class II haplotype, as well as co-stimulatory proteins on the antigen-presenting cell such as B7-1 and B7-2, have roles in regulation of the differentiation of naive T helper cells into specific Th subsets \[^{513,514}\]. However, the best characterised factors affecting the development of Th subsets are cytokines themselves. For example, IFN-\(\gamma\) inhibits the differentiation and effector functions of Th2 cells, and can lead to a dominant Th1 response. The APC-derived cytokine IL-12 strongly drives the differentiation of Th1 cells \textit{in vitro} and \textit{in vivo}, partly through its potent induction of IFN-\(\gamma\) production. Conversely, IL-4 strongly directs the development of Th2 cells, both \textit{in vitro} and \textit{in vivo}, and mice in which the IL-4 gene has been disrupted have an impaired ability to generate Th2 responses. Furthermore, IL-4, IL-10 and IL-13 inhibit Th1-cell proliferation, and oppose the effects of IFN-\(\gamma\) on macrophages. Therefore, reciprocal regulation occurs between the Th1- and Th2-cell subsets.

\textbf{Fig. 1.27} Development of Th1 and Th2 cells from uncommitted precursor T cells. (Reproduced from Cytokine bulletin, R&D Systems, Summer 1995, with permission).
1.8.(B).2 The Th1 & Th2 paradigm in autoimmunity

Polarised Th2 responses have been implicated in the pathogenesis of systemic autoimmune diseases that are experimentally induced by allogeneic interactions (i.e., chronic stimulatory graft-versus-host disease, host-versus-graft disease) or chemicals (mercury model, gold and D-penicillamine models) \(^{506,515}\). Goldman and colleagues concluded that the preferential activation of Th2 cells could explain some common features of these models, such as MHC Class II hyperexpression on B cells, hyper-IgE, increased IL-4 activity and impairment of IL-2 production.

However, in the majority of autoimmune diseases, particularly the organ specific ones, Th1-type responses have been implicated. The majority of CD4\(^+\) T cell clones isolated from lymphocytic thyroid infiltrates of patients with Hashimoto’s thyroiditis or Graves’ disease had a clear cut Th1 lymphokine profile with production of high TNF-\(\alpha\) and IFN-\(\gamma\) concentrations, and such clones exhibited cytolytic potential \(^{516,517}\). Feldmann’s group, also demonstrated Th1 characteristics in (thyroid peroxidase) TPO-specific T cell clones \(^{132,133}\). In 1993, a quite homogeneous Th1 profile was also observed in CD4\(^+\) T cell clones derived from retro-ocular infiltrates of patients with TAO \(^{392}\). Also, in patients with multiple sclerosis (MS) most clones derived from both peripheral blood (PB) and cerebrospinal fluid (CSF) showed a Th1 profile \(^{518,519}\). Adoptive transfer experiments in experimental autoimmune encephalomyelitis (EAE; an inflammatory autoimmune disease of the central nervous system which serves as an animal model for MS) recently demonstrated that myelin basic protein (MBP-) or proteolipid protein (PLP-) specific Th1 clones could induce disease, whereas Th2 clones specific for the same peptide-MHC complexes could not \(^{520,521}\). In insulin-dependent diabetes mellitus (IDDM) evidence
suggesting a pathogenetic role for Th1 cells has recently been provided by independent groups using different animal models of the disease [522,523]. Lately, exceptions have started to emerge. Feldmann’s group demonstrated that Th2 cells predominated in TSH-R-specific T cell clones, using autoantigen transfected EBV-transformed B-cell lines [132,133].

Also, analysis of diabetic rats has suggested a protective role for Th2 cells [524,525]. In 1994, Scott et al. demonstrated that BALB/c T cells derived from the double transgenic TCR-HNT/Ins-HA mouse model for the disease (expressing influenza haemagglutinin [HA], on islet β cells [Ins-HA], and a T cell receptor transgene [TCR-HNT] specific for a class II-restricted HA peptide) produced high levels of IFN-γ, as well as elevated and sustained levels of IL-4 [526]. Since the Th2 promoting activity of IL-4 has been shown to be dominant over the Th1-promoting cytokines IL-12 and IFN-γ [527] the data are consistent with a dominant BALB/c genetic predisposition towards Th2 differentiation, which confers resistance to spontaneous autoimmune diabetes. Although effector CD4⁺ T cells appear to be irreversibly committed to one given Th subset, the generation of Th1 or Th2 effector cells from naive or memory CD4⁺ T cells appears to be dependent on the cytokine milieu at the time of the antigenic challenge. Therefore, therapeutically changing the cytokine profile of autoreactive CD4⁺ T cells (at the population level) might be feasible and may have great potential in the treatment of autoimmune inflammatory disorders; one of the current goals of such therapies being the careful “long term” upregulated production of immunosuppressive cytokines at sites of inflammation.
In this report, I have studied the ultrastructural localisation of GAGs in the extraocular muscles of patients with TAO. Also, I have investigated the measurement of specific serum and urinary GAGs, as possible indicators of disease activity.

**1.8.1 Structure of GAGs**

GAGs are the major chemical polymers of the ground substance and connective tissue (in association with collagen, reticulin and elastin). Nomenclature tends to be confusing since the terms mucopolysaccharide (related to the physical properties of the solutions of GAGs), mucoprotein, mucin, mucus and glycoprotein have been used synonymously. The position however, was clarified by Jeanloz \(^{[528]}\), who introduced the term *glycosaminoglycan*. This, more clearly defines the chemical nature of these compounds as being high molecular weight (ranging from 13-30 kDa) polymers with repeating dimers of an amino-sugar usually linked to a hexuronic acid. The amino-sugar (hexosamine) is either a glucosamine or a galactosamine, both of which are usually either acetylated or sulphated at the amino-group and both of which may also be sulphated either at carbon 4 or carbon 6 of the hexose ring. The hexuronic acid is either glucuronic acid or iduronic acid. The term *glycosaminoglycan* is now a generic term for hyaluronic acid, chondroitin sulphate, dermatan sulphate, heparan sulphate, keratan sulphate, and heparin (Fig. 1.28, page 125-127). Table 1.9, (page 126) summarises the nomenclature and structure of these GAGs.
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Fig. 1.28 (a)

HYALURONIC ACID

Fig. 1.28(b)

CHONDROITIN 4/6-SULPHATE

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**Fig. 1.28(c)**

**DERMATAN SULPHATE**

**Fig. 1.28(d)**

**HEPARAN SULPHATE**

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Fig. 1.28(e) Molecular structures of (a) hyaluronic acid, (b) chondroitin sulphate, (c) dermatan sulphate, (d) heparan sulphate, (e) keratan sulphate, and (f) heparin.
### Table 1.9 Nomenclature of GAGs (adapted from Jeanloz\(^{528}\))

<table>
<thead>
<tr>
<th>Modern Systematic Name</th>
<th>Old Name</th>
<th>Hexosamine</th>
<th>Hexuronic Acid</th>
<th>Hexosamine Sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin</td>
<td>Chondroitin</td>
<td>GalNAc</td>
<td>GlcUA</td>
<td>None</td>
</tr>
<tr>
<td>Chondroitin-4- sulphate</td>
<td>Chondroitin sulphate A</td>
<td>GalNAc</td>
<td>GlcUA</td>
<td>O-SO(_4)</td>
</tr>
<tr>
<td>Chondroitin-6- sulphate</td>
<td>Chondroitin sulphate C</td>
<td>GalNAc</td>
<td>GlcUA</td>
<td>O-SO(_4)</td>
</tr>
<tr>
<td>Dermatan sulphate</td>
<td>Chondroitin sulphate B</td>
<td>GalNAc</td>
<td>GlcUA</td>
<td>O-SO(_4^*)</td>
</tr>
<tr>
<td></td>
<td>Heparin</td>
<td>GlcNAc</td>
<td>GlcUA</td>
<td>N-SO(_4^*)</td>
</tr>
<tr>
<td></td>
<td>Heparin sulphate</td>
<td>GlcNAc</td>
<td>GlcUA</td>
<td>N-SO(_4^*)</td>
</tr>
<tr>
<td></td>
<td>Heparin monosulphate</td>
<td>GlcNAc</td>
<td>GlcUA</td>
<td>O-SO(_4^*)</td>
</tr>
<tr>
<td>Keratan sulphate I</td>
<td>Corneal keratosulphate</td>
<td>GlcNAc</td>
<td>None**</td>
<td>O-SO(_4)</td>
</tr>
<tr>
<td>Keratan sulphate II</td>
<td>Skeletal keratosulphate</td>
<td>GlcNAc</td>
<td>None**</td>
<td>O-SO(_4)</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>Hyaluronic acid</td>
<td>GlcNAc</td>
<td>GlcUA</td>
<td>None</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:**  
GlcNAc= N acetyl glucosamine; GalNAc= N acetylgalactosamine;  
GlcUA= glucuronic acid; IdUA= iduronic acid

* Iduronic acid may also be sulphated at C-2

** Galactose replaces hexuronic acid
Fig. 1.29 *Diagrammatic representation of proteoglycans aggregate*. Hyaluronic acid acts as a filamentous backbone for making non-covalent associations with several proteoglycan subunits (consisting of chondroitin sulphate and keratan sulphate chains attached to a protein core) to form giant proteoglycan aggregate\textsuperscript{[529]}. (Reproduced with permission, from reference\textsuperscript{[529]}, © 1975, by Academic Press, Inc).
With the probable exception of hyaluronic acid \(^{530,531}\), all of the GAGs occur in the native state covalently bound at the reducing end to specific proteins to form proteoglycans \(^{532-539}\) (formerly called mucoproteins) (Fig. 1.29). The polyanionic GAGs radiate out from the central core protein much like bristles on a brush and, because of their high-charge density, form an extended structure \textit{in vivo}. Moreover, electrostatic repulsion of their negatively charged groups (carboxyl and sulphate groups) tends to keep the chains separate from each other. However, they are free to sweep through the surrounding environment so that the volume occupied by a molecule in solution is several-fold greater than the partial specific volume of the dehydrated molecule. A number of either carboxyl- or amino- linked oligosaccharides can be present on the same protein backbone thus rendering the proteoglycans even more complex and versatile. This structure gives the tissues resilience and enables them to maintain their characteristic anatomical form.

\textbf{1.8.(C).2 Biosynthesis of GAGs (proteoglycans) in connective tissues and control of their production}

The biosynthesis of connective tissue GAGs, the proteoglycans included, takes place inside the fibroblasts \(^{540}\). Cells other than those present in connective tissue, e.g., lymphocytes and leukocytes, also synthesise the same proteoglycans \(^{541}\). Cytokines have been shown to stimulate fibroblasts to secrete GAGs or proliferate \(^{423,542}\). Inside the cell, GAGs, with the possible exception of hyaluronic acid, are synthesised by stepwise transfer of monosaccharides (from the corresponding nucleotide sugars) to a core protein. The core protein, which needs to be formed prior to the monosaccharide transfer, is synthesised by established routes...
for protein synthesis on the ribosomes. The GAGs present extracellularly in connective tissue are first synthesised intracellularly and then exported outside the cells via the Golgi apparatus, when they appear close to the surface and subsequently spread into the extracellular space where they form the gel-like ground substance. The average half life of GAG chains in humans is thought to be the same as in experimental animals - approximately 21/2 days.

1.8.(C).3 Biological functions of GAGs

Dorfman in 1958 first suggested the role of GAGs in biological processes including control of water and electrolytes in extracellular fluids, lubrication of joints, wound healing, calcification, blood coagulation, hair growth, cell proliferation, blood-lipid clearing activity and as a refractive medium in the eye. More recently, several other biological functions of GAGs have come to light. These include an important role in the regulation of cell proliferation, cell-to-matrix binding, regulation of diffusion and of flow of macromolecules through tissue, growth factor attachment, cell-to-cell interaction and regulation of interleukin-1 production in local inflammatory response.

All of these functions of GAGs and proteoglycans are attributable to their large hydrodynamic volume and their propensity to interact with other molecules through ionic interactions. The large hydrodynamic volume of GAGs means that they bind a large amount of water and occupy a large space relative to their weight. Therefore, they are likely to fill most of the intercellular space, thus providing an extracellular carbohydrate-water milieu. Glycosaminoglycans, however, do much more than fill space; they interact with many other molecules. Most extracellular matrix proteins and many growth factors have binding sites for GAGs. The
extracellular matrix proteins with such binding sites include fibronectin \(^{[547]}\), the interstitial collagens \(^{[548]}\), laminin \(^{[549]}\), vitronectin \(^{[550]}\), and thrombospondin \(^{[551]}\).

Among the growth factors, the various forms of fibroblast and endothelial cell growth factors in particular have very high affinities for glycosaminoglycans \(^{[552,553]}\).

The decisive factor in the interactions of GAGs seems to be their charge density due to the presence of the sulphate and carboxyl groups on these macromolecules.

**1.8.(C).4 The role of hyaluronan in TAO**

Most of the signs and symptoms of TAO are largely explicable by the enlargement of the EOMs and the retro-ocular fat/connective tissue within the confined non-expansile cavity of the orbit. GAGs are likely to play a major role in this process by increasing the volume of the affected tissues.

Biochemical analysis of retro-bulbar connective tissue identified hyaluronic acid (51\%) and dermatan sulphate (DS; 30\%) as the two major fractions, whereas chondroitin-4-sulphate and chondroitin-6-sulphate, heparan sulphate and heparin were only found in small amounts \(^{[554]}\).

Hyaluronic acid is noted for its ability to bind water because of its large size and its large hydrodynamic specific volume, both of which give rise to a high entropy of dilution, permitting binding up to 500 ml of water per gram of hyaluronic acid \(^{[555,556]}\). In some pathological conditions water retention and rigidity of a connective tissue, like skin, is increased due to increased concentration of hyaluronic acid. Such is the case in localised myxoedema, in which skin hyaluronic acid increases 6-16 times \(^{[416]}\).

Histological studies of retro-bulbar connective tissue specimens, taken from patients with TAO, showed an accumulation of GAGs along with infiltration of
immunocompetent cells in these specimens. However, detailed studies of the ultrastructural immunolocalisation of hyaluronic acid at the EOM level have not been performed as yet.

### 1.8. (C).5 GAG turnover in TAO

In an adult approximately 250 mg of GAGs are disposed of daily. Of these only 3-6 mg are excreted in the urine per day while the remainder are degraded in the body. Peptides containing chondroitin sulphate are taken up by the liver, whereas heparin and heparan sulphate are cleared by the vascular endothelium. Peptide-free GAG-chains in the blood are preferentially cleared by renal excretion. This excretion constitutes an estimated fraction of less than 1/10 of the GAGs released from the intercellular matrix.

Since therapy for TAO is different in active and inactive disease, it would be highly desirable to have a marker for the activity of the disease. It has been suggested that the level and composition of GAGs present in serum and urine could provide easily accessible indicators of the activity of TAO. In 1968, Winand found the urinary excretion of chondroitin sulphates A and C, keratan sulphate, and heparan sulphate to be increased approximately 2-fold in patients with severe TAO, as assessed by colorimetric analysis. In the same study, total serum GAG concentrations were elevated 3-fold, and the values were unrelated to thyroid function. Kahaly et al. have also demonstrated a possible increase in urine and serum GAG levels in early active TAO by photometric quantitation of hexuronic acids after reaction with carbazole. However, a Japanese study of serum hyaluronan levels in patients with TAO, which employed a radioimmunoassay, has failed to detect values significantly different from those obtained for the control cases studied. The
radioimmunoassay employed by the Japanese study apparently enables direct measurement of serum hyaluronan with a much higher degree of accuracy whereas colorimetric assays by their nature are not very accurate. In addition, the low sensitivity of colorimetry requires concentrations of the samples in multiple steps. Therefore, the above mentioned studies are not directly comparable. However, the Japanese study results are somewhat surprising since accumulation of GAGs including hyaluronan in affected tissues is a major pathological change in TAO\cite{416}. Therefore the technique they employed warrants re-examination with a larger group of TAO patients and ideally with a range of disease activity. The finding of possible increase in urinary GAGs is of interest, since this would provide a convenient way of measuring disease activity in serial samples. It therefore justifies a further study assessing urinary GAGs not only quantitatively but qualitatively as well, in order to ascertain whether a specific urinary GAG pattern is related to TAO in the first place and to TAO activity in particular.
Chapter 2

Adhesion molecule expression *in vivo*

on

extraocular muscles

in

thyroid-associated ophthalmopathy
2.1 INTRODUCTION

TAO is an autoimmune condition characterised by mononuclear cell infiltration of the EOMs and/or the orbital fat/connective tissue with associated deposition of GAGs in the interstitial spaces. The binding of leukocytes to endothelial cells is an essential initial step in the inflammatory response, it promotes leukocyte trafficking and is mediated by adhesion molecules.

The one previous study of in situ expression of selectin and immunoglobulin superfamily type adhesion molecules in TAO has utilised retro-ocular connective tissue specimens obtained from three patients with severe TAO undergoing orbital decompression surgery [291], as EOM tissue is not excised during the course of orbital decompression surgery for TAO. However, in the same study EOM tissue was obtained at autopsy, from one patient with severe TAO, and strong ICAM-1 immunoreactivity was detected on the perimysial tissue but not on the EOMs themselves [291].

To provide further information on the expression of adhesion molecules in TAO we have taken biopsies of affected EOMs from two groups of TAO patients. The first group consisted of patients with early, active TAO, and the second with late, inactive TAO. The pattern of adhesion molecules ICAM-1, E-Selectin, V-CAM as well as that of the leukocyte integrins CD11a/CD18, CD11b/CD18, and CD11c/CD18 was assessed by means of immunohistochemistry.
Table 2.1. Early, active TAO patient characteristics

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/Sex</th>
<th>Duration of TAO</th>
<th>Class at time of biopsy</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>54/F</td>
<td>6 m</td>
<td>4(4b)</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>39/F</td>
<td>6 m</td>
<td>4(4b,2b)(^\d)</td>
<td>R(^g),S(^*) (prior to biopsy)</td>
</tr>
<tr>
<td>3</td>
<td>42/F</td>
<td>4 m</td>
<td>4(4b)</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>29/M</td>
<td>3 m</td>
<td>4(4b,2b)</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>51/F</td>
<td>5 m</td>
<td>4(4b)</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>52/M</td>
<td>6 m</td>
<td>4(4b,2b)</td>
<td>R(^g),S(^*) (prior to biopsy)</td>
</tr>
</tbody>
</table>

\(^a\) Duration of TAO: as expressed the time elapsed since diagnosis, ys: years, m: months
\(^b\) Class according to modified NO SPECS classification\(^{[193]}\)
\(^c\) Class prior to biopsy: 6(6a,4b,2b)
\(^d\) R: Orbital radiotherapy, * S: Steroids
2.2 PATIENTS AND METHODS

2.2.1 Patients

EOM biopsies were obtained from 2 patient groups with TAO. All patients were biochemically euthyroid at the time of biopsy and class 4 according to modified NO SPECS classification\(^{[255]}\), (page 39). Informed consent and Ethical Committee approval were obtained for both patient groups. The biopsies were obtained from the belly of the muscle (10-12 mm from the insertion), the muscle sheath having been dissected off, and without prior application of any diathermy.

(a) Early TAO. Six biopsies were taken under local anaesthesia from 6 patients with early disease not undergoing corrective strabismus surgery (see Table 2.1). Patients nos 2 and 6 still showed evidence of active orbital inflammation despite having had treatment with radiotherapy followed by steroids. No complications were encountered during this procedure and none of these patients developed ocular motility problems as a result of their biopsy.

(b) Late TAO. Thirteen biopsies were collected from 11 late, inactive TAO patients (see Table 2.2, next Page). Patients nos 8 and 9 were euthyroid from the onset of TAO and at the time of biopsy. Specimens were taken during the course of corrective strabismus surgery for tight EOMs, which were restricting normal ocular movements.

(c) Controls. Twelve normal-appearing EOM biopsies obtained during routine non-thyroid related strabismus surgery (7 males, 5 females; age 24-55 years, mean age 43) served as controls.
### Table 2.2. Late, inactive TAO patient characteristics

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>Duration of TAO*</th>
<th>Class prior to biopsy†</th>
<th>Class at time of biopsy†</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>47/M</td>
<td>9 ys</td>
<td>4(4b)</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>52/M</td>
<td>10 m</td>
<td>4(4b,2b)</td>
<td>R* (prior to biopsy)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>66/M</td>
<td>9 ys</td>
<td>4(4b)</td>
<td>S§ (prior to biopsy)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>55/F</td>
<td>20 m</td>
<td>4(4b)</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>40/M</td>
<td>17 m</td>
<td>4(4b)</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>57/F</td>
<td>10 m</td>
<td>4(4b,2b)</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>69/F</td>
<td>12 m</td>
<td>6(6a,4b)</td>
<td>R‡ (prior to biopsy)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>67/M</td>
<td>40 m</td>
<td>6(6a,4b)</td>
<td>R‡, S§ (prior to biopsy)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>60/F</td>
<td>30 m</td>
<td>4(4b)</td>
<td>R‡, S§ (prior to biopsy)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>61/M</td>
<td>40 m</td>
<td>4(4b)</td>
<td>S§ (prior to biopsy)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>56/M</td>
<td>27 m</td>
<td>6(6a,4b)</td>
<td>R‡, S§ (prior to biopsy)</td>
<td></td>
</tr>
</tbody>
</table>

*Duration of TAO: as expressed the time elapsed since diagnosis, ys: years, m: months
†Class according to modified NO SPECS classification
‡R: Orbital radiotherapy, §S: Steroids
2.2.2 Immunohistochemical studies

All EOM specimens were embedded in optimal cutting temperature (OCT) embedding medium, snap-frozen in liquid-nitrogen and stored at -70 °C until used. Transverse cryostat sections (5μm) were mounted on 3-amino propyl tri-ethoxy silane (APES, Sigma, Poole, UK)-coated slides. Consecutive cryostat sections were immunostained with monoclonal antibodies to the studied adhesion molecules and haematoxylin and eosin (H&E). Primary antibody binding was visualised using an avidin-biotin system. All antibodies (anti-ICAM-1, anti-ELAM-1, anti-VCAM-1, anti-CD11a, anti-CD11b, anti-CD11c) were obtained from R&D Systems Europe (Abingdon, UK), except Ulex lectin (Sigma.) and UCHL1 (Dako, High Wycombe, UK).

Slides were thawed at room temperature and air-dried for 30 minutes. Before immunohistochemical staining, sections were fixed in cold acetone (4 °C) for 5 minutes and washed in phosphate buffered saline (PBS; pH 7.4) for another 15 mins. Immunoperoxidase staining was performed using the Vector ABC Kit (PK-4002; Vector Laboratories, Peterborough, UK) with a few modifications. Non-specific antibody uptake was blocked using normal horse serum for 20 mins, followed by incubation with a primary monoclonal antibody (MoAb) for 30 mins in a humidified chamber at room temperature. After a wash in PBS for 10 mins the secondary biotinylated horse anti-mouse Ab was applied for 30 mins. Endogenous peroxidase activity was suppressed by incubating sections in 0.3% H₂O₂ in 50% methanol for 20 mins. After a further wash in PBS for 10 mins, sections were incubated with a preformed avidin and biotinylated horseradish peroxidase macromolecular complex for 45 mins. The peroxidase reaction was then developed with the chromogenic
Table 2.3. Mean percentage of blood vessels (by comparison with ulex lectin) or infiltrating mononuclear cells (x 400 field) expressing adhesion molecules in the EOMs of early, late TAO and control specimens

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Percent positive vessels</th>
<th>Percent positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ulex Lectin</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>Early TAO (n=6)</td>
<td>100.00</td>
<td>89.00***</td>
</tr>
<tr>
<td></td>
<td>+/- SEM</td>
<td>0.00</td>
</tr>
<tr>
<td>Late TAO (n=13)</td>
<td>100.00</td>
<td>42.23***</td>
</tr>
<tr>
<td></td>
<td>+/- SEM</td>
<td>0.00</td>
</tr>
<tr>
<td>Normal EOM (n=12)</td>
<td>100.00</td>
<td>2.83</td>
</tr>
<tr>
<td></td>
<td>+/- SEM</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*** (p<0.001) n=number of samples
Statistical analysis refers to comparisons between samples from TAO and normal EOM using the same Ab.

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substrate amino-ethyl-carbazole (AEC; Sigma). The substrate was filtered and 30% hydrogen peroxide (Sigma) was added to a final concentration of 0.03% H$_2$O$_2$. Sections were developed for 2 to 5 mins, washed in distilled water, and counterstained in Mayer’s haematoxylin for 30 seconds. Finally, sections were washed in cold tap water for 5 mins, mounted with aqueous-based glycerol gelatine medium (DAKO) and covered with a glass coverslip. The primary antibody was omitted to provide a negative control, and palatine tonsil tissue sections from tonsillectomies were used to confirm optimal staining concentration for Abs used. Positively-stained cells or vessels were counted per x400 microscope field, by two separate observers (myself and Annette Bacon) blinded to the origin of specimen, and the mean result from at least 9 fields was calculated.

**2.2.3 Statistical analysis**

Student’s t-test was carried out on the immunohistochemistry data with SPSS software, and $p$ value <0.05 was considered significant in the comparisons between control and disease samples.

**2.3 RESULTS**

**2.3.1 Immunohistochemistry**

(a) Normal EOM. Examination of normal appearing EOM biopsies from non-TAO patients showed that maximum 3% of the vessels stained faintly with ICAM-1, 2% with VCAM-1, and 1% very faintly with E-selectin (by comparison with ulex lectin). Very few T cells were found in control specimens and those present expressed minimal levels of the integrins studied (Table 2.3, page 139).

(b) Early TAO. Strong ICAM-1 immunoreactivity was detected in the interstitial and perimysial connective tissue that surrounds EOM fibres. In addition to
its expression in connective tissue cells, prominent ICAM-1 immunoreactivity was also detected in numerous mononuclear cells and in ulex lectin positive vascular endothelial cells (Fig 2.1, Fig 2.2, page 142). There was marked up-regulation of ICAM-1 (89%, *p*<0.001), and E-selectin (83%, *p*<0.001) as well as VCAM-1 (85%, *p*<0.001) (Table 2.3, page 139) (Fig 2.3, page 143). In counting CD11a⁺, CD11b⁺, CD11c⁺ cells it was observed that CD11a (Fig 2.4, page 143) was significantly up-regulated (72%, *p*<0.001), as well as CD11b (46%, *p*<0.001) and CD11c (32%, *p*<0.001). EOM fibres themselves did not demonstrate ICAM-1 immunoreactivity. In both early and late TAO EOM specimens, faint VCAM-1 immunoreactivity was also detected in occasional connective tissue cells that did not show any ulex lectin immunoreactivity.

(1c) Late TAO. Although the distribution of adhesion molecules was the same in both early and late TAO specimens, there was a striking difference in the levels of expression of these molecules. In spite of the fact that the endothelial markers in the early TAO specimens reached close to maximal levels of expression, in the late TAO specimens only 25-40% of ulex lectin-positive vessels expressed ICAM-1, ELAM-1 or VCAM-1 (Table 2.3, page 139). Correspondingly, lower levels of the counterpart ligands for ICAM-1 were observed on the infiltrating T cells in late TAO specimens (Table 2.3, page 139).
Fig 2.1 Light micrograph of EOM tissue from a strabismus control patient. Immunoperoxidase staining for ulex lectin on a cryostat section. Positive staining of capillary endothelium seen as a brown precipitate (arrows). (x476)

Fig 2.2 Light micrograph of immunoperoxidase staining for intercellular adhesion molecule-1 (ICAM-1) on a cryostat section of EOM tissue from a patient with TAO. There is irregular staining of the endomysium of individual muscle fibres (arrows) seen as a brown precipitate. (x476)
Fig 2.3 Light micrograph of immunoperoxidase staining for endothelial leucocyte adhesion molecule-1 (ELAM-1) (E-selectin) on a cryostat section of EOM tissue from a patient with TAO. There is positive staining of the vascular endothelium (arrows). (x476)

Fig 2.4. Light micrograph of immunoperoxidase staining for CD11a on a transversely cut cryostat section of extraocular muscle from a TAO patient. Several mononuclear cells show positive staining of their cytoplasmic membranes (arrows). (x476)
2.4 DISCUSSION

This study demonstrated, by immunohistochemistry, that ICAM-1, ELAM-1, VCAM-1, and the leukocyte integrins (CD11a/CD18, CD11b/CD18, CD11c/CD18) were minimal or absent in normal appearing EOM biopsies harvested from control patients. In contrast, in early, untreated TAO specimens, strong ICAM-1 immunoreactivity was observed in the interstitial and perimysial connective tissue surrounding EOM fibres, in numerous mononuclear cells, and in ulex lectin-positive vascular endothelial cells. Also, immunoreactivity for ELAM-1 and VCAM-1 was detected at high levels in ulex lectin-positive blood vessels in the early TAO specimens studied. However, immunoreactivity for the same vascular adhesion molecules in late TAO specimens only reached levels of 25-40% of ulex lectin-positive vessels, although the distribution of these adhesion molecules was the same in both early and late TAO specimens. Concerning the leukocyte integrins (CD11a/CD18, CD11b/CD18, CD11c/CD18), again significantly higher levels of expression were detected in the early TAO specimens compared with late TAO specimens. It should be noted that in this study clinically active disease was present in the early disease patients.

EOM fibres themselves had no immunoreactivity for the adhesion molecules studied, suggesting that it is the extracellular matrix component of the EOMs which is involved in the inflammatory process. The lack of staining on the EOM fibres is in agreement with preliminary observations by Heufelder and Bahn [291] on EOM tissue from one patient with severe TAO. In conclusion, the immunohistochemistry results suggest a strong correlation between increased expression of adhesion molecules and disease activity.

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Heufelder and Bahn have demonstrated previously that IFN-γ, tumour necrosis factor-alpha (TNF-α) and IL-1α are capable of inducing the expression of functionally active ICAM-1 in cultured retro-ocular fibroblasts \cite{363}. Also, the same group have demonstrated that immunoreactivity for IFN-γ, TNF-α and IL-1α can be detected in vascular endothelium and connective tissue of retro-ocular tissue biopsies obtained from patients with severe Graves' ophthalmopathy, but not in retro-ocular tissue from normal individuals \cite{395}. Recently, we have shown that EOM-derived T cell lines from TAO patients are capable of producing a panel of cytokines including IFN-γ, TNF-β and transforming growth factor-beta (TGF-β) \cite{561}. It is thus possible, that certain cytokines, present in the EOM vasculature microenvironment, are involved in the activation of vascular adhesion molecules such as ICAM-1, ELAM-1 and VCAM-1, and facilitate the recruitment and access of certain immunocompetent cells to the EOMs in TAO.

At least some of these adhesion molecules could be potential targets for future therapeutic intervention. Given the currently increasing understanding of the way these various receptors and their counter-receptors work together \textit{in vivo} in regulating the normal physiology of EOMs and the way they contribute to the pathogenesis of TAO, the challenge now is to discover ways of inhibiting \textit{in vivo} these interactions in a monospecific, non-toxic way. The use of soluble forms of these receptors or their counter-receptors to control the disease process, as well as the use of multivalent ligand constructs as antagonists of the endothelial adhesion molecules are currently being explored.
Chapter 3

Analysis of extraocular muscle infiltrate

in vivo

in

thyroid-associated ophthalmopathy
3.1 INTRODUCTION

In most T cell-mediated autoimmune diseases, activated T cells are found infiltrating the target tissue and there is raised expression of HLA Class II antigens in disease sites. In surgical specimens from patients with autoimmune thyroid disease the thyroid follicular cells show aberrant expression of HLA-DR antigens, which potentially enables them to present autoantigen on their cell surfaces to infiltrating T cells \(^{[22,147]}\). A similar mechanism has been suggested in TAO, where orbital antigens are targets for infiltrating T cells.

The identity of the primary target in the orbit is still unknown although both the EOM fibre and orbital fibroblast have been proposed as candidates \(^{[562-564]}\). Orbital tissue is not easily available for immunohistopathological analysis and, in the majority of cases, EOM surgery is not indicated until late in the course of the disease. Most of the EOM samples studied previously have come from patients either with longstanding disease, or from those who had been on long-term immuno-suppressive therapy \(^{[290,296,298]}\).

In the present study, we have analysed 17 EOM biopsies from 14 patients with both early, active and late, inactive TAO using a panel of monoclonal antibodies to identify the subtypes of infiltrating cells present and the expression of HLA-DR on the muscle fibres and other cells. By examining the involved EOMs at different stages of disease, we aimed to clarify the distribution of Class II expression and to increase our understanding of the types, subtypes and activation markers of cells infiltrating the EOMs during the active stage of disease.
### Table 3.1. Early, active TAO patient characteristics

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/Sex</th>
<th>Duration of TAO</th>
<th>Class at time of biopsy</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65/M</td>
<td>5 m</td>
<td>4(4b)</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>57/M</td>
<td>5 m</td>
<td>4(4b)</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>61/F</td>
<td>1 m</td>
<td>4(4b,2b)</td>
<td>S** (prior to biopsy)</td>
</tr>
<tr>
<td>4</td>
<td>53/F</td>
<td>6 m</td>
<td>4(4b,2b)</td>
<td>S**, A‡ (prior to biopsy)</td>
</tr>
<tr>
<td>5</td>
<td>42/M</td>
<td>3 m</td>
<td>4(4b)</td>
<td>None</td>
</tr>
</tbody>
</table>

* Duration of TAO: expressed as the time elapsed since diagnosis, m: months

† Class according to modified NO SPECS classification\(^{[193]}\)

** S: Steroids

‡ Azathioprine
### 3.2 PATIENTS AND METHODS

#### 3.2.1 Patients

EOM biopsies were obtained from 2 patient groups with TAO. All patients were biochemically euthyroid at the time of biopsy and class 4 according to modified NO SPECS classification\(^{[193]}\). Informed consent and Ethical Committee approval was obtained for both patient groups. EOM enlargement was confirmed on computed tomography\(^{[195]}\) or ultrasound scanning\(^{[194]}\). The biopsies were obtained from the belly of the muscle (10-12 mm from the insertion), the muscle sheath having been dissected off, and without prior application of any diathermy.

(a) **Early TAO.** Five EOM biopsies were taken from five patients with recent onset of disease (see Table 3.1) who had a mean duration of symptoms of four months. In three cases biopsy of the inferior rectus was performed under topical anaesthesia (amethocaine 1%). No complications were encountered during this procedure and none of these patients developed ocular motility problems as a result of their biopsy. The remainder of the specimens were obtained under general anaesthesia during orbital decompression surgery. Patients nos 3 and 4 still showed evidence of active orbital inflammation despite having had treatment with steroids (no 3), steroids and azathioprine (no 4).

(b) **Late TAO.** Twelve biopsies were collected from 9 late, inactive TAO patients (see Table 3.2, next page). Patients nos 7 and 12 were euthyroid from the onset of TAO and at the time of biopsy. Specimens were taken during the course of corrective strabismus surgery for tight EOMs, which were restricting normal ocular movements.
<table>
<thead>
<tr>
<th>Patient no</th>
<th>Age/Sex</th>
<th>Duration of TAO*</th>
<th>Class prior to biopsy</th>
<th>Class at time of biopsy</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>55/M</td>
<td>3ys+9m</td>
<td>4(4b)</td>
<td></td>
<td>R⁺ (prior to biopsy)</td>
</tr>
<tr>
<td>7</td>
<td>57/M</td>
<td>13 ys</td>
<td>4(4b)</td>
<td></td>
<td>S§ (11 ys prior to biopsy)</td>
</tr>
<tr>
<td>8</td>
<td>57/F</td>
<td>3 ys</td>
<td>4(4b)</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>40/M</td>
<td>9 ys</td>
<td>4(4b)</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>71/F</td>
<td>14 ys</td>
<td>4(4b)</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>47/M</td>
<td>6 ys+4m</td>
<td>4(4b)</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>12</td>
<td>55/F</td>
<td>21 ys</td>
<td>4(4b,2b)</td>
<td></td>
<td>S§ (prior to biopsy)</td>
</tr>
<tr>
<td>13</td>
<td>40/M</td>
<td>3 ys+6m</td>
<td>4(4b)</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>14</td>
<td>67/M</td>
<td>2 ys+10m</td>
<td>6(6a,4b)</td>
<td>4(4b)</td>
<td>R⁺, S§ (2 ys prior to biopsy)</td>
</tr>
</tbody>
</table>

* Duration of TAO: expressed as the time elapsed since diagnosis, ys: years, m: months
† Class according to modified NO SPECS classification
‡ R: Orbital radiotherapy, § S: Steroids
(c) Controls. Sixteen normal-appearing EOM biopsies from 14 patients either undergoing routine non-thyroid related strabismus surgery (n=13) or enucleation for intraocular malignancy (n=1) provided the control specimens. In 2 control patients 2 EOMs were biopsied. Patients’ ages ranged from 6-59 years (mean age: 32.6 years).

### 3.2.2 Immunohistochemical studies

All EOM specimens were embedded in optimal cutting temperature (OCT) embedding medium, snap-frozen in liquid-nitrogen and stored at −70 °C until used. Transverse cryostat sections (5µm) were mounted on 3-amino propyl tri-ethoxy silane (APES; Sigma, Poole, UK)-coated slides. Consecutive cryostat sections were immunostained with monoclonal antibodies to the studied inflammatory infiltrate and haematoxylin and eosin (H&E). Primary antibody binding was visualised using an avidin-biotin system and the Vector ABC Kit (PK-4002; Vector Laboratories, Peterborough, UK), (for method see section 2.2.2, page 138).

The MoAbs used in this study were as follows: CD45RB (leucocyte common antigen, clone PD 7/26, dilution 1:50); CD45RO (pan T cell, clone UCHL1, dilution 1:50); CD20 (B cell, clone L26, dilution 1:50); CD4 (CD4 T cells, clone MT310, dilution 1:10); CD8 (CD8 T cells, clone DK25, dilution 1:50); MAC (macrophages and activated monocytes, clone Ber-MAC3, dilution 1:100); HLA-DR (MHC Class II, clone CD3/43, dilution 1:100); von Willebrand factor (capillary endothelial cells, dilution 1:200). All monoclonal antibodies were obtained from DAKO except for von Willebrand factor which was obtained from Serotec (Kidlington, Oxford, UK).

The primary antibody was omitted to provide a negative control, and palatine tonsil tissue sections from tonsillectomies were used to confirm optimal staining.
concentration for Abs used. Positively-stained cells were counted per x400 microscope field, by two independent researchers blinded to origin of specimen, and the mean result from at least 9 fields was calculated.

### 3.2.3 Statistical analysis

Mann Whitney U - Wilcoxon Rank Sum W test was carried out on the immunohistochemistry data with SPSS software, and $p$ value <0.001 was considered significant in the comparisons between control and disease samples.

### 3.3 RESULTS

#### 3.3.1 Immunohistochemistry

**(1a) Normal EOM.** Examination of normal appearing EOM biopsies from non-TAO patients showed that these contained few CD45RO$^+$ cells, with less than 1 cell counted per field (Table 3.3, next page). Cells were found throughout the tissue and in a perivascular distribution. Equal numbers of CD4$^+$ and CD8$^+$ cells were present (mean 0.39 compared with 0.43 cells per field counted respectively).

Practically no CD20$^+$ cells were found. Macrophages were observed in all of the control specimens, situated in the interstitial tissue and adjacent to the muscle fibres. The muscle fibres themselves did not stain with any of the monoclonal antibodies used, including that to HLA-Class II antigens although interstitial cells did stain positively with Dako-DR CR3/43. Capillary endothelial cells were also DR$^+$. 

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Table 3.3. Mean cell counts (x400 field) of positive cells in the EOMs of early, late TAO and control specimens

<table>
<thead>
<tr>
<th>Specimens</th>
<th>CD45RB</th>
<th>CD45RO</th>
<th>CD20</th>
<th>CD4</th>
<th>CD8</th>
<th>MAC</th>
<th>HLA-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early TAO (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cell count</td>
<td>35.36</td>
<td>19.30</td>
<td>3.60</td>
<td>9.54</td>
<td>8.08</td>
<td>13.96</td>
<td>29.44</td>
</tr>
<tr>
<td>+/- SEM</td>
<td>23.75</td>
<td>13.17</td>
<td>3.50</td>
<td>8.08</td>
<td>7.16</td>
<td>6.22</td>
<td>13.44</td>
</tr>
<tr>
<td>Late TAO (n=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cell count</td>
<td>4.70</td>
<td>3.17</td>
<td>0.59</td>
<td>1.28</td>
<td>0.86</td>
<td>6.17</td>
<td>24.04</td>
</tr>
<tr>
<td>+/- SEM</td>
<td>0.60</td>
<td>0.75</td>
<td>0.25</td>
<td>0.40</td>
<td>0.41</td>
<td>1.29</td>
<td>4.30</td>
</tr>
<tr>
<td>Normal EOM (n=16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cell count</td>
<td>2.35</td>
<td>0.92</td>
<td>0.23</td>
<td>0.39</td>
<td>0.43</td>
<td>3.18</td>
<td>15.20</td>
</tr>
<tr>
<td>+/- SEM</td>
<td>0.34</td>
<td>0.14</td>
<td>0.05</td>
<td>0.11</td>
<td>0.13</td>
<td>0.78</td>
<td>2.55</td>
</tr>
</tbody>
</table>

n=number of samples
Table 3.4. Statistical analysis of mean cell counts (x400 field) of positive cells in the EOMs of early, late TAO and control specimens using the Mann-Whitney U - Wilcoxon Rank Sum W test.

<table>
<thead>
<tr>
<th>Specimens</th>
<th>CD45RB</th>
<th>CD45RO</th>
<th>CD20</th>
<th>CD4</th>
<th>CD8</th>
<th>MAC</th>
<th>HLA-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early TAO (n=5)</td>
<td>18.50</td>
<td>19.00</td>
<td>10.00</td>
<td>16.50</td>
<td>13.30</td>
<td>18.20</td>
<td>13.00</td>
</tr>
<tr>
<td>Normal EOM (n=16)</td>
<td>8.66</td>
<td>8.50</td>
<td>11.31</td>
<td>9.28</td>
<td>10.28</td>
<td>8.75</td>
<td>10.38</td>
</tr>
<tr>
<td>p value</td>
<td>0.0004</td>
<td>0.0001</td>
<td>0.7190</td>
<td>0.0194</td>
<td>0.3539</td>
<td>0.0012</td>
<td>0.4451</td>
</tr>
<tr>
<td>Late TAO (n=12)</td>
<td>19.58</td>
<td>19.50</td>
<td>15.50</td>
<td>17.75</td>
<td>14.42</td>
<td>18.25</td>
<td>17.17</td>
</tr>
<tr>
<td>Normal EOM (n=16)</td>
<td>10.69</td>
<td>10.75</td>
<td>13.75</td>
<td>12.06</td>
<td>14.56</td>
<td>11.69</td>
<td>12.50</td>
</tr>
<tr>
<td>p value</td>
<td>0.0037</td>
<td>0.0044</td>
<td>0.59</td>
<td>0.0735</td>
<td>0.9818</td>
<td>0.0373</td>
<td>0.1457</td>
</tr>
</tbody>
</table>

n=number of samples
Statistical analysis refers to comparisons between samples from TAO and normal EOM using the same Ab.
(1b) Early TAO. The extraocular muscle fibres were histologically intact. Two biopsies obtained under local anaesthesia were small with few muscle fibres and infiltrating cells present (patients 2 and 5). Early disease specimens contained increased numbers of all cell types compared with specimens from patients with late TAO and from control patients. In particular an increase in CD45RO⁺ (Fig. 3.1), CD4⁺, CD8⁺, and MAC⁺ cells (Fig. 3.2, next page) were observed in the interstitial tissue. The EOM biopsy from patient 3, who had the shortest and most acute history, was heavily infiltrated with CD45RO⁺ cells although, unlike the other EOM specimens, CD20⁺ cells were also found. In general, the early and late disease EOM had similar numbers of DR⁺ cells (mean 29.44 in early TAO, mean 24.04 in late TAO) (Table 3.3, page 153) (Fig. 3.3, next page). The EOM fibres, from all patients with early TAO, were negative for DR. Capillary endothelial cells, which were identified with von Willebrand factor, were DR⁺. The numbers of CD45RO⁺, CD45RB⁺, and MAC⁺ cells in early EOM biopsies were statistically significant compared with those in the control specimens (p<0.001; Table 3.4).

Fig 3.1 Light micrograph of a transversely cut extraocular muscle tissue from a patient with TAO. Immunoperoxidase staining for CD45RO on a cryostat section. Positive staining is seen as a brown precipitate (arrowed). (x476)
Fig 3.2 Light micrograph of immunoperoxidase staining for macrophages on a cryostat section of extraocular muscle biopsy from a patient with TAO (brown precipitate; arrowed). (x476)

Fig 3.3 Light micrograph of immunoperoxidase staining for HLA class II (DR) on a cryostat section of extraocular muscle tissue from a patient with TAO. Positively stained cells and their processes are located in the endomysium and perimysium (brown precipitate; arrowed). (x476).
(1c) Late TAO. Cells positive for CD45RB, CD45RO, MAC, and DR were found in smaller numbers in the EOM from patients with longstanding disease (Table 3.3, page 153). CD45RO\(^+\) cells were seen scattered throughout the tissue and in a perivascular distribution. These specimens contained few CD20\(^+\) cells, with less than one cell counted per field. The EOM fibres did not stain with any of the MoAbs.

### 3.4 DISCUSSION

In our study we have compared EOMs from patients with both recent-onset and longstanding disease. Although orbital fat and levator muscle from the upper lid have been studied from patients with active disease \(^{[297]}\), EOMs from patients with acute disease is rarely available for analysis \(^{[298]}\). In three cases with early disease, who would not otherwise have undergone surgery, we biopsied one inferior rectus muscle under local anaesthesia. Although the small sizes of the specimens and the patchy distribution of disease make quantitative analyses difficult, the examination of EOM from patients with recent TAO is of value in attempting to clarify the underlying mechanisms as they reflect what is occurring in the muscles early in the course of the disease.

Our findings in longstanding TAO are similar to those reported in previous studies, with some T cells present and an increase in HLA Class II expression \(^{[290,296]}\). In the specimens from patients with early TAO a predominantly T cell (CD4\(^+\) and CD8\(^+\)) infiltrate was observed and few CD20\(^+\) B cells were found in early TAO and even fewer in late TAO specimens. Previous histopathological studies of involved EOMs have reported lymphocytic infiltration of muscle with
both B and T cells, with macrophages present in variable numbers \cite{288,294-296,415}. Deposition of GAGs by fibroblasts in the interstitial spaces between the muscle fibres results in enlargement of EOMs \cite{416}. Although the enlargement of the EOMs is one of the most prominent features in TAO, in our study the muscle fibres themselves were morphologically intact, in keeping with previous findings \cite{288,294}.

Increased expression of HLA-DR was detected in early disease and was also raised in late disease compared with control specimens. However, the muscle fibres in all specimens remained negative for HLA Class II. This finding is in contrast to that reported by Hiromatsu et al. in 1995 \cite{298}. They reported that HLA-DR expression was detected on EOM fibres in 4 out of 38 patients \cite{298}. This discrepancy may be due to different patient characteristics. The interstitial cells in our disease specimens also expressed HLA Class II antigens as did capillaries which may be of importance in the homing of lymphocytes. Other markers of early activation such as the expression of CD69 \cite{565} in early, clinically active disease specimens would be very interesting to examine in subsequent studies.

Normal extraocular muscle has been reported to contain a few T cells, mostly of T cell suppressor type, and macrophages in the interstitial spaces \cite{297,566}. In 1989, Schmidt and co-workers \cite{566} found that the cells were distributed evenly along the length of the extraocular muscle. Our control specimens showed a similar picture with a small number of T cells, macrophages, but, practically no B cells. Cells staining for HLA-DR were also found in the interstitial tissue and resembled fibroblasts morphologically. We did not find a predominance of CD4$^+$ or CD8$^+$ cells in our control EOM specimens unlike van der Gaag (1993) \cite{179} but, this may be due to the smaller size of our specimens. Skeletal muscle, however, has been noted to contain similar numbers of CD4$^+$ and CD8$^+$ cells \cite{567}. We did not find any
age-related changes with regard to CD8^+ T cells.

Of particular interest is the significant increase in macrophages in early, and less so in late, disease compared with control tissue. Macrophages are found in normal EOM and, as professional antigen-presenting cells, may play a role in immune mechanisms. In 1993, van der Gaag and co-workers[^297] reported that the inferior and medial recti of normal individuals contained the greatest number of macrophages. This finding has been put forward to explain the increased frequency of clinical involvement in these muscles. However, as the inferior rectus and inferior oblique muscles also have an extensive and well developed connective tissue system, this is an alternative explanation for the frequent clinical involvement of the inferior rectus[^568]. In addition to the presence of macrophages, normal EOM contained HLA-DR expression localised to the capillary endothelial cells. These findings are in agreement with those reported by Hiromatsu *et al.*, in 1995[^298], who also demonstrated a lack of correlation between DR expression and clinical severity. One possible explanation for DR expression in normal tissue could be as part of an immune surveillance mechanism.

The HLA-DR^+ cells detected in diseased EOMs may be a result of T cell activation and release of cytokines such as interferon-γ, an important inducer of HLA Class II expression, which may be involved in the perpetuation of the disease. When stimulated by T cells fibroblasts produce GAGs[^569] which result in enlargement of the EOMs in acute TAO by osmotically attracting water, and thus causing protrusion of the eyeballs and optic nerve compression in severe disease[^253]. Further proliferation of fibroblasts, which could be independent of the infiltrating T cells, might be responsible for the later fibrosis and scarring of the EOMs associated with double vision[^570].
In TAO the lack of an animal model and the relative inaccessibility of the orbit have restricted investigation. The majority of patients do not require surgical intervention until the disease is quiescent when specimens obtained would be expected to show only non-specific end-stage changes. The study of circulating lymphocytes in the peripheral blood, although of value, may not accurately reflect what is occurring in the target tissue \(^{303,385}\). Results in this study have demonstrated that inflammatory cells (CD4\(^+\) and CD8\(^+\)) are present in early TAO together with an increased number of macrophages. Increased expression of HLA-DR by interstitial cells was observed in both early and late TAO, suggesting a role for autoimmune mechanisms in mediating this disease. With increased understanding of the underlying pathogenesis it may become possible to intervene at an earlier stage and prevent the complications of this distressing condition.
Chapter 4

Analysis of extraocular muscle-infiltrating T cells in thyroid-associated ophthalmopathy
4.1 INTRODUCTION

TAO is characterised by an autoimmune process affecting the orbital contents. T cells have been demonstrated, by us and others, in affected EOMs\textsuperscript{290,294} and although massive lymphocytic infiltration is unusual, varying numbers of activated T cells and macrophages can be seen present in biopsies. Tumour necrosis factor-\(\beta\) (TNF-\(\beta\)), interleukin-1\(\alpha\) (IL-1\(\alpha\)) and interferon-\(\gamma\) (IFN-\(\gamma\)) have been shown to be present in affected retro-ocular connective tissue\textsuperscript{395} and are able to induce GAG production by fibroblasts\textsuperscript{399} in culture, which may explain the pathogenesis.

Most previous studies of T cell immune responses to putative TAO antigens\textsuperscript{303,387,571} have used peripheral blood T cells, which may or may not reflect the responses of cells within the target organ. Such studies of T cell responses from the site of disease have been limited by inaccessibility of the affected tissues, the relative rarity of TAO warranting operative intervention, and the small amount of tissue available for study. More recent studies have utilised T cells isolated from specimens, particularly orbital fat, obtained during decompressive surgery of the orbit\textsuperscript{393,405}, since this is the most accessible source of orbital tissue.

In this study, biopsies of affected EOMs and peripheral blood were taken from TAO patients during the course of corrective strabismus surgery, to examine the phenotype and cytokine profile of the infiltrating T cells. The EOM-infiltrating T cells and peripheral blood T cells were isolated and expanded \textit{in vitro} and their phenotype determined by FACS analysis. Cytokine production was then assessed using ELISA techniques on the supernatants of mitogen-stimulated T cell lines. In the second part of the study, the pattern of cytokine gene expression in EOMs was studied \textit{ex vivo} in biopsies taken from a further five TAO patients,
Table 4.1 TAO patient characteristics (Patients no. 1-5 were used for generating T cell lines; patients no. 6-10 for study of cytokine profiles in whole tissue)

<table>
<thead>
<tr>
<th>Pat. no.</th>
<th>Age</th>
<th>Sex</th>
<th>Class** of TAO</th>
<th>T4</th>
<th>Other treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70</td>
<td>F</td>
<td>4(4b)</td>
<td>Yes</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>M</td>
<td>4(4b,2b)</td>
<td>No</td>
<td>R* (prior to biopsy)</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>M</td>
<td>6(6a,4b)</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>F</td>
<td>4(4b,2b)</td>
<td>No</td>
<td>S† (prior to biopsy)‡</td>
</tr>
<tr>
<td>5</td>
<td>38</td>
<td>F</td>
<td>4(4b)</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>M</td>
<td>4(4b,2b)</td>
<td>Carbimazole</td>
<td>S† (prior to biopsy)</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>F</td>
<td>6(6a,4b,2b)</td>
<td>Yes</td>
<td>S† (prior to biopsy)§</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>F</td>
<td>4(4b)</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>63</td>
<td>F</td>
<td>4(4b,2b)</td>
<td>No</td>
<td>S† (prior to biopsy)</td>
</tr>
<tr>
<td>10</td>
<td>47</td>
<td>M</td>
<td>6(6a,5a,4c,2c)</td>
<td>No</td>
<td>R*, S† (prior to biopsy)§</td>
</tr>
</tbody>
</table>

** Class of TAO is according to modified NO SPECS classification [193]

* R: Radiotherapy; † S: Steroids; ‡ At biopsy receiving Prednisolone 5 mg/day;
§ At biopsy receiving Prednisolone 7.5 mg/day
and results were compared with the data derived from the T cell lines. Because of small biopsy size, the same specimens could not be used for generation of lines and cytokine gene expression studies. Finally, the antigenic reactivity of two biopsy-derived T cell lines was determined using proliferation assays in the presence of thyroid extract, EOM extract, and a TSH receptor (TSHR) preparation.

4.2 PATIENTS AND METHODS

4.2.1 Patients

Extraocular muscle biopsies were collected from TAO patients (with their informed consent and Ethical Committee approval) undergoing corrective strabismus surgery. These biopsies were obtained from the belly of the muscle (10-12 mm from the insertion), with the muscle sheath having been removed and without prior application of any diathermy. TAO patient details are given in Table 4.1. In addition, 5 normal appearing EOM biopsies were obtained during routine non-thyroid related strabismus surgery from three patients (2 males, 1 female; age 4-55 years) who served as controls.

4.2.2 Reagents and Antibodies

Normal heat-inactivated human AB serum (HS) from a single male donor was obtained from Sigma (Poole, UK; lot no. 53 HO790). Fetal calf serum (FCS) was purchased from Gibco (Paisley, UK). Recombinant human IL-2 (hrIL-2) was obtained from Boehringer-Mannheim (Lewes, UK). Phytohaemagglutinin-L (PHA-L; leucockagglutinin), and Dutch-modified RPMI 1640 medium were purchased from Sigma. Fluorochrome-coupled MoAbs against CD3, CD4, CD8, CD45RO, CD14, CD25, HLA-DR, HLA-DQ (Leu 10), α/β and γδ TCR were obtained from Becton
Dickinson (Oxford, UK); appropriate, isotype-matched control mAbs were obtained from Becton Dickinson or Southern Biotechnology Associates (Birmingham, AL, USA).

All procedures were performed at room temperature (20°C) unless otherwise stated.

**4.2.3.(a) Peripheral blood**

Heparinised peripheral whole blood samples were layered over Ficoll-Isopaque solution and the porous filter disc of the LeucoSep® System (Greiner Labortechnik Ltd, Dursley, UK) and then spun at 1000 g for 10 minutes. Following this, mononuclear cells were carefully recovered by a sterile glass Pasteur pipette from the plasma / Ficoll-Isopaque interface into 20 mls of 10% human serum (HS) complete medium and the remaining Ficoll-Isopaque discarded along with the red cell pellet. The mononuclear cells were centrifuged at 250 g for 10 mins. The supernatant was discarded, the pellet resuspended and then washed twice in 10% HS complete medium, centrifuging at 250 g for 10 mins to precipitate the cells between each wash. The cells were then counted using a Neubauer haemocytometer. Cell viability was assessed by diluting the cells 1:1 in 0.4% trypan blue (Sigma, UK). Dead cells stained blue while viable cells remained unstained. Following this the cells were either used immediately or cryopreserved.

**4.2.3.(b) EOM biopsies and cell lines**

Biopsied extraocular muscle tissue was placed immediately into 2 ml of 12.5% complete medium, consisting of 12.5% (v/v) human serum in Dutch modified RPMI 1640 medium supplemented with 2mM L-glutamine, 1mM sodium pyruvate,
1% nonessential amino acids and gentamicin (50μg/ml; Sigma).

The biopsy was then rinsed twice in 12.5% complete medium, under sterile conditions, in order to remove any contaminating blood and placed in a 24-well tissue culture plate (Nunc; Gibco) in 2 mls of 12.5% complete medium with PHA-L 1μg/ml and human recombinant hrIL-2 50U/ml and incubated at 37°C in humidified, 5% CO2-in-air atmosphere for 3 days. Following this, two-thirds of the medium was replaced with fresh medium containing hrIL-2 50 U/ml.

By day 7, lymphoblasts which had migrated out of the biopsy could be observed and were restimulated with fresh medium, PHA and hrIL-2. Cells were restimulated by the weekly addition of irradiated (2500 cGy) peripheral blood mononuclear cells (PBMCs) from the same patient, at a ratio of 2:1 PBMCs/T cells. Peripheral blood T cell lines from the same patient were developed in parallel to those from the EOMs, and used as a control for the EOM-derived T cell lines. PBMCs were separated from fresh heparinised blood by Ficoll-Hypaque (Pharmacia, St. Albans, UK) density gradient centrifugation, cultured at 5x10^5 cells/ml in 2-ml wells in 12.5% complete medium, and stimulated with PHA and hrIL-2 in a weekly cycle as described above. At no time were the lines cultured with specific antigen.

4.2.4 Immunofluorescence staining

Cell samples were washed in cold PBS with 1% HS (pH 7.2-7.4) and phycoerythrin (PE)-, peridinin chlorophyll protein (PerCP)- and fluorescein isothiocyanate (FITC)-conjugated MoAbs were added to 1-2 x 10^5 cells at 4°C, and incubated on ice for 45 min. Following two washes in 2 ml of cold PBS, cells were resuspended in PBS and analysed immediately. Staining patterns of lymphocytes (5000 events), gated by forward and side scatter, were analysed on a FACScan flow
cytometer (Becton Dickinson) using Lysis II and Paint-a-Gate analysis programs.

### 4.2.5 Cytokine production by T cell lines

T cells were cultured at $10^6$/ml in 2-ml volumes of 12.5% complete medium containing PHA-L (1µg/ml), and supernatants were harvested at 24, 48 and 72 hours, residual cells were pelleted by centrifugation and the supernatant stored at -20 °C until assayed with specific sandwich ELISA kits (R&D Systems Europe Ltd, Abingdon, UK). Supernatants from all the T cell lines were assayed for IL-2, IFN-γ, TNF-β, IL-4, IL-10, IL-12, and transforming growth factor-β1 (TGF-β1). Supernatants were assayed in duplicate, undiluted and at dilutions of 1:10 and 1:20.

### 4.2.6 Antigens

**(6a) Human EOM antigen preparation.** Human EOM tissue was obtained from a patient who underwent orbital exenteration for a poorly differentiated squamous cell carcinoma arising in the upper lid conjunctiva, without evidence of EOM involvement. The tissue was minced and homogenised with a motor homogeniser for 5x10 seconds in 5 ml 15mM Tris/2 mM MgCl₂ pH 7.5 (Solution A), with protease inhibitors (PEFABlock; Sigma), freeze/thawed twice and centrifuged for 30 min at 40,000 g, 4 °C. The pellet was resuspended in 1ml 75 mM Tris pH 7.5; MgCl₂ 12.5mM; ethylenediaminetetraacetic acid (EDTA) 0.3mM; ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) 1mM; 250mM sucrose; 1mM phenylmethylsulphonyl fluoride (PMSF); 1µM PEFAblock (4-(2-aminoethyl) benzene-sulphonyl fluoride hydrochloride) (Solution B). To solubilise, 1ml of membranes in solution B were added to 4 mls (Tris 7.5 10mM, NaCl 50mM, 1% Triton), and homogenised by two to three strokes in a glass/glass homogeniser. The
solution was kept on ice for 1 hour, homogenising every 10-15 min. The mixture was centrifuged for 2 h at 100,000 g, 4 °C, and the supernatant was used as a source of antigen. The MicroBCA Protein Assay Kit (Pierce & Warriner, Chester, U.K.) was used to determine the protein concentration of each of the preparations, which were stored at −70 °C until used, with prior irradiation (4000 cGy of γ-irradiation from a 237Cs source). Both EOM membranes and extracts were used at final concentrations of 10, 1 and 0.1 µg/ml for the proliferation assays.

(6b) Human thyroid gland antigen preparation. Non-nodular thyroid gland tissue was obtained from a patient who underwent thyroidectomy for an enlarging nodule. The tissue was minced with scissors and forceps in 5 ml of Solution A, homogenised and subsequently processed and treated as for the EOM tissue (see above) to yield both EOM membranes and solubilised antigen preparations. Both thyroid gland tissue membranes and extracts were used at final concentrations of 10, 1 and 0.1 µg/ml for the proliferation assays.

(6c) Production and purification of TSHR fusion protein. The TSHR antigen preparation was the extra-cellular domain (ECD) of the TSHR expressed as a maltose binding protein (MBP) fusion, as previously described [572], and it was the kind gift of Dr. Marian Ludgate, Department of Pathology, University of Wales College of Medicine. Before use the antigen was reconstituted with filter-sterilised distilled water (Sartorius, Epsom, UK) and used at final concentrations of 10, 1 and 0.1 µg/ml.

### 4.2.7 Proliferation assays

Proliferative responses to the previously mentioned antigenic preparations were performed using 5x10^4 antigen-presenting cells (APCs; irradiated autologous...
PBMCs) which were incubated with \(2 \times 10^4\) T cells in 200 µl of 12.5% complete medium for 72 hours. Assays were performed in triplicate using round-bottomed 96-well culture plates (Nunc). The T cells were assayed at least 10 days after the last antigen stimulation with PHA and at least five days after the last addition of IL-2. Non-specific cell proliferation was measured in wells containing T cells alone, and all assays included a positive control of T cells plus PHA (1 or 0.1 µg/ml) and APCs. After 72 hours at 37 °C in an atmosphere of 5% CO\(_2\) in air, cultures were pulsed for the last 6 hours with 1 µCi/well of methyl \(^3\)H-thymidine (Amersham, Aylesbury, UK) and harvested onto glassfibre filters (Whatman, Maidstone, UK) using a Minimash 2000 cell harvester (Dynex Technologies, Billingshurst, UK). \(^3\)H-thymidine incorporation in the harvested glassfibre filters was measured by liquid scintillation counting. Results are expressed as a stimulation index (SI): mean ct/min incorporated in the presence of antigen divided by mean ct/min incorporated in its absence. An SI of 2 or more was considered as a positive response.

### 4.2.8 RNA extraction from EOM samples

Total RNA was extracted from the EOM biopsies (patients nos 6-10) by homogenisation of 1-2 mg biopsy specimens in 1 ml of TRIazon\(^2\) (Gibco) according to the manufacturer's protocol, as detailed elsewhere \[^{573}\]. Briefly, lysates were transferred to microfuge tubes and 200 µl of chloroform added. Samples were vortexed for 1 min, incubated at room temperature for 2 min and centrifuged at 11 000 g for 15 min at 4 °C. The aqueous phase containing the RNA was transferred to a clean tube, mixed with 0.5 ml of isopropyl alcohol (Fisons, Loughborough, UK),
and placed at -20 °C for 2-3 hours. Precipitated RNA was pelleted by centrifugation
at 11 000 g for 15 min, washed once with 1 ml of 75% ethanol, vacuum-dried for 2-3
min, and resuspended in 30 µl diethylpyrocarbonate-treated deionised water.
Samples were used immediately to synthesise cDNA or stored at -70 °C.

### 4.2.9 cDNA synthesis

Using SuperScript II reverse transcriptase (SS-RT; Gibco), cDNA was
synthesised in a 40 µl of RT buffer (50 mM Tris pH 8.3, 75 mM KCl, 3 mM MgCl₂),
containing 2 µl oligo (dT) primers (500 µg/ml) (Gibco), 10mM dithiothreitol (DTT),
400 units SS-RT (Gibco), 1 unit RNAguard RNAase inhibitor, 0.5 mM
deoxynucleotide triphosphate (dATP, dGTP, dCTP, dTTP in equal concentrations;
Promega, Southampton, UK), and 1 µg of sample RNA.

After a 50-min incubation period at 42 °C, the samples were incubated for another 15
min at 70 °C to stop cDNA synthesis, cooled to 4 °C and stored at -20 °C.
Table 4.2. Oligonucleotide primers used for PCR amplification of cytokine cDNA, and β-actin cDNA reported as the sense (S) primer followed by the antisense (AS) primer

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Oligonucleotide primers</th>
<th>DNA product size (bp)</th>
<th>Ref. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>5'-GTCTCTTGAATGCCAGAATCTGCTCTATC-3' 5'-CATGTCAAATTCTCATGCTCCATC-3'</td>
<td>420</td>
<td>[574]</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-AAACAGATGAGGTGCTCTTTCCAGG-3' 5'-TGGAGAAGCAACACTTGGTCTCCA-3'</td>
<td>388</td>
<td>[574]</td>
</tr>
<tr>
<td>IL-2</td>
<td>5'-GAATGGAATTTAATTACAAGAATCCC-3' 5'-TGTTTCAGATCCCTTTAGTCCAG-3'</td>
<td>222</td>
<td>[575]</td>
</tr>
<tr>
<td>IL-4</td>
<td>5'-GTCTCACCTCCCAACTGCTTC-3' 5'-CTTGTGCTGGATGAACTGCTG-3'</td>
<td>299</td>
<td>[575]</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-CTTCGGTGCTGTTCTTCCTCAACTGCTC-3' 5'-GCTCTGCTGCTGTTCCT-3'</td>
<td>402</td>
<td>[575]</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-TTGGCAAGCCTTCCTGATTTA-3' 5'-AACTTCTCCAACACCTCCTG-3'</td>
<td>247</td>
<td>[576]</td>
</tr>
<tr>
<td>IL-10</td>
<td>5'-ATGCCCCAAGCTGAGAACAAGCACA-3' 5'-TCTCAAGGGGCTGGTGACGTATCCCA-3'</td>
<td>352</td>
<td>[575]</td>
</tr>
<tr>
<td>IL-12 (p40)</td>
<td>5'-TACTCTCTTGTGCTCCCTCTG-3' 5'-GTGGCCATAGGGAACCTGAG-3'</td>
<td>500</td>
<td>[178]</td>
</tr>
<tr>
<td>IL-13</td>
<td>5'-GAAGACCCAGAGTGCT-3' 5'-GGGCCATAGGGAAGACCT-3'</td>
<td>257</td>
<td>[178]</td>
</tr>
<tr>
<td>IL-15</td>
<td>5'-CAAGACTGATATTGATAGGA-3' 5'-ACATTGGCAATAGTACAAA-3'</td>
<td>557</td>
<td>[178]</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-CTGATTTTCACTTCTTGGCT-3' 5'-TATCCGCTCACATGGAATGA-3'</td>
<td>180</td>
<td>[575]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-CAAGGGGAAGATCCCTCCAG-3' 5'-CTTGTGCTGTTAGGAACG-3'</td>
<td>325</td>
<td>[574]</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-GTGGGGCGCCAGGAAGACG-3' 5'-CTTCCTTATGTCCAGCAGATTTCC-3'</td>
<td>534</td>
<td>[576]</td>
</tr>
<tr>
<td>TCR</td>
<td>5'-GTGACAAATGCTGCTGCTACA-3' 5'-CAAAGCCTTTCTGACCA-3'</td>
<td>261</td>
<td></td>
</tr>
</tbody>
</table>

Anastasia Pappa  
PhD Thesis
4.2.10 Polymerase chain reaction amplification

Primer sequences for polymerase chain reaction (PCR) were as published elsewhere \[^{[178,574-576]}\]. The primer sequences and the predicted size of the amplified PCR fragments are shown in Table 4.2. PCR amplification was carried out, as previously described \[^{[573]}\]. As a positive control for PCR amplification, reverse transcribed RNA isolated from PBMCs, which were cultured in RPMI 1640 containing 10% FCS and stimulated with PHA-M (PHA; Gibco), 1 \(\mu\text{g/ml}\), for 24 h, was included with each PCR assay. As a positive control for IL-12 and IL-15 probes, adherent PBMCs were also cultured for 4 hours with lipopolysaccharide (LPS; 10 \(\mu\text{g/ml}\); Sigma); RNA was extracted from the two PBMC preparations, pooled together and used to prepare cDNA as described above. In each assay control reactions without cDNA were carried out in parallel, to ensure that reagents were not contaminated. Additional controls, in which reverse transcription was omitted, were also performed in order to detect any possible amplification of spurious products derived from contamination of RNA samples with genomic DNA.

4.2.11 Oligonucleotide hybridization

Product identification was confirmed by hybridization studies, as described previously \(^{[178,573]}\) using probes detailed in Table 4.3 (next page)

4.2.12 Statistical analysis

Student's \(t\)-test was carried out on the proliferation assay data with SPSS software, and \(p < 0.05\) was considered significant in the comparisons between SIs from EOM- and PBMC-derived T cell lines using the same antigenic preparation.
Table 4.3 Oligonucleotide probes used for PCR hybridisation

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Oligonucleotide probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>5'-TCTTCCTCTGTAGTCATTGGCGATGGCCTC-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-GGCAGCTCCGACCTACGAA-3'</td>
</tr>
<tr>
<td>IL-2</td>
<td>5'-CAGTTAAGACCCAGGGA-3'</td>
</tr>
<tr>
<td>IL-4</td>
<td>5'-GCTAGCATGTGCAGCAGCA-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-GGGCTGAGATGCGAGGATGTCGCGAA-3'</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-CAGGGACTCTTCAAAAACCTCTCCACAACCC-3'</td>
</tr>
<tr>
<td>IL-10</td>
<td>5'-CGGCGCTGTCATCGATT-3'</td>
</tr>
<tr>
<td>IL-12</td>
<td>5'-CAGTACTAGTTGACATTCAG-3'</td>
</tr>
<tr>
<td>IL-13</td>
<td>5'-CGCAAAAGGTCTCAGCTG-3'</td>
</tr>
<tr>
<td>IL-15</td>
<td>5'-ACCGTGGCTTTGAGTAAT-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-GGGTTTTCTAGCTTGCA-3'</td>
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<tr>
<td>TNF-α</td>
<td>5'-CCAGGCAGTCAGGATCAG-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CCGGAGATCCATACGATGCGCTGTGAGA-3'</td>
</tr>
<tr>
<td>TCR</td>
<td>5'-AGTGCTGTGCAGCAGCA-3'</td>
</tr>
</tbody>
</table>

4.3 RESULTS

4.3.1 Culture of EOM infiltrating lymphocytes

EOM biopsies were necessarily small (~2mm³) and the number of lymphocytes was estimated, by light microscopy, to be < 30 by day 3 of culture. Blast cells were seen within a week, and although the numbers initially were small, they exceeded 10⁶ cells by 6-10 weeks of culture. Lines were established successfully from all biopsies and PBMCs.
### Table 4.4. FACS® Analysis of EOM-biopsy and PBMC derived T cell lines from four patients with TAO

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell line</td>
<td>PB1</td>
<td>B1.1</td>
<td>PB2</td>
<td>B2.1</td>
<td>PB3</td>
<td>B3.2</td>
<td>B3.3</td>
<td>PB4</td>
<td>B4.1</td>
</tr>
<tr>
<td>EOM biopsied</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3⁺ (%)</td>
<td>100</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>CD45RO⁺ (%)</td>
<td>100</td>
<td>99</td>
<td>99</td>
<td>98</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>CD3⁺ CD4⁺ (%)</td>
<td>23</td>
<td>95</td>
<td>98</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>85</td>
<td>89</td>
<td>12</td>
</tr>
<tr>
<td>CD3⁺ CD8⁺ (%)</td>
<td>57</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>68</td>
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<tr>
<td>CD3⁺ TCR α/β⁺ (%)</td>
<td>93</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>98</td>
<td>95</td>
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<tr>
<td>CD3⁺ TCR γδ⁺ (%)</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CD25⁺ HLA-DR⁺ (%)</td>
<td>46</td>
<td>67</td>
<td>99</td>
<td>52</td>
<td>99</td>
<td>86</td>
<td>48</td>
<td>37</td>
<td>21</td>
</tr>
<tr>
<td>HLA-DR⁺ (%)</td>
<td>53</td>
<td>95</td>
<td>99</td>
<td>97</td>
<td>99</td>
<td>89</td>
<td>94</td>
<td>35</td>
<td>80</td>
</tr>
<tr>
<td>HLA-DQ⁺ (%)</td>
<td>2</td>
<td>23</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

Cells were stained with FITC-conjugated Abs against CD8, TCR α/β, HLA-DQ, PE-conjugated Abs against CD3, CD25, CD45RO, TCR γδ, and PERCP-conjugated Abs against CD3, CD4, HLA-DR. Results represent the percentage of positive cells in the different lines.

PB: Peripheral Blood  B: Biopsy  LIR: Left Inferior Rectus  RIR: Right Inferior Rectus
4.3.2 Phenotyping of expanded EOM infiltrating T lymphocytes and PBMC-derived T cell lines

Ten EOM biopsy T cell lines from five TAO patients were established using this procedure. In six EOM biopsy-derived T cell lines from four TAO patients, sufficient cells were obtained for full FACS analysis. PBMC T cell lines (as a control) were developed in parallel from each patient and expanded in the same way as the biopsy T cell lines. Two colour FACS analysis of these lines is shown in Table 4.4. Five of these EOM T cell lines were found to be >85% CD3⁺ CD4⁺. All were TCR α/β⁺ and none expressed the γδ receptor. However, in one biopsy 68% of CD3⁺ cells were CD8⁺ (patient no. 4). All PBMC- and EOM-derived lines were negative for CD14 (monocytes) and CD19 (B cells), while all 6 EOM-derived T cell lines were CD45RO⁺. All lines showed evidence of activation when stained with relevant markers such as HLA-DR and CD25. The expression of HLA-DQ molecules was not detected on five of the EOM-derived T cell lines; in the other, 23% of the cells were HLA-DQ⁺. Similar to the EOM-derived T cell lines, PBMC T cell lines were mainly CD4⁺ (three out of four were >89% CD3⁺ CD4⁺) and TCR α/β⁺. Again one predominantly CD8⁺ T cell line (57% CD3⁺ CD8⁺) (patient no.1) was observed, but not in the same patient as had the CD8⁺ EOM-derived T cell line.
### Table 4.5. Cytokine release by EOM-biopsy and PBMC derived T cell lines at 24 hours after stimulation

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
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<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>Patient 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB1</td>
<td>783</td>
<td>1164</td>
<td>109</td>
<td>145</td>
<td>316</td>
<td></td>
</tr>
<tr>
<td>B1.1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td><strong>Patient 2</strong></td>
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<td></td>
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</tr>
<tr>
<td>PB2</td>
<td>&lt;31.3</td>
<td>778</td>
<td>&lt;7.8</td>
<td>14</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td>B2.1</td>
<td>&lt;31.3</td>
<td>931</td>
<td>96</td>
<td>&lt;7</td>
<td>202</td>
<td></td>
</tr>
<tr>
<td>B2.2</td>
<td>32</td>
<td>330</td>
<td>9</td>
<td>64</td>
<td>273</td>
<td></td>
</tr>
<tr>
<td><strong>Patient 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB3</td>
<td>106</td>
<td>1026</td>
<td>&lt;7.8</td>
<td>9</td>
<td>223</td>
<td></td>
</tr>
<tr>
<td>B3.1</td>
<td>NA</td>
<td>68</td>
<td>NA</td>
<td>&lt;7</td>
<td>262</td>
<td></td>
</tr>
<tr>
<td>B3.2</td>
<td>877</td>
<td>1788</td>
<td>1516</td>
<td>9</td>
<td>314</td>
<td></td>
</tr>
<tr>
<td>B3.3</td>
<td>NA</td>
<td>383</td>
<td>NA</td>
<td>16</td>
<td>193</td>
<td></td>
</tr>
<tr>
<td>B3.4</td>
<td>NA</td>
<td>218</td>
<td>NA</td>
<td>9</td>
<td>171</td>
<td></td>
</tr>
<tr>
<td>B3.5</td>
<td>NA</td>
<td>162</td>
<td>NA</td>
<td>285</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td><strong>Patient 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB4</td>
<td>43</td>
<td>270</td>
<td>36</td>
<td>&lt;7</td>
<td>184</td>
<td></td>
</tr>
<tr>
<td>B4.1</td>
<td>36</td>
<td>422</td>
<td>&lt;7.8</td>
<td>&lt;7</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>B4.2</td>
<td>&lt;31.3</td>
<td>574</td>
<td>136</td>
<td>&lt;7</td>
<td>286</td>
<td></td>
</tr>
</tbody>
</table>

NA: *Not assessed*

*48 hours

Mean values presented, with SEM <10% of the mean.
4.3.3 Cytokine release by EOM- and peripheral blood-derived T cell lines

Nine biopsy-derived T cell lines and four peripheral blood lines were investigated for cytokine production by ELISA. The majority released IL-4 (60% biopsy and 75% PBMC lines), IFN-γ (all lines), IL-10 (100% biopsy and 50% PBMC lines), TNF-β (56% biopsy and 75% PBMC lines), TGF-β1 (all lines), and IL-2 (all lines), (Table 4.5). No IL-12 production was detected suggesting that there was not any contamination of the T cell lines by other cells such as dendritic cells or monocytes at that time stage.

4.3.4 Antigenic recognition by EOM- and PBMC-derived T cell lines

Adequate numbers of cells of both biopsy and PBMC T cell lines, as well as sufficient autologous APCs, were available from two individual patients for antigen presentation studies. Dose-dependent proliferation was observed with a biopsy-derived T cell line from patient no. 3, in response to the thyroid membranes and extracts (maximum SIs of 6 and 19, respectively). Responses were also seen to EOM membranes and extracts (Table 4.6, page 179; Table 4.7, page 181). In several samples the proliferation response was not dose-dependent and this may be explained by a toxicity of the antigenic preparation at high concentrations. Another EOM biopsy-derived T cell line from patient no. 5 showed no specific proliferation, indicating that the antigens used had no non-specific stimulating activity to account for these results.

Ideally PBMC-derived T cell lines from healthy individuals would be required as controls. Also, regarding the TSH-R preparation further experiments using the same expression system for the preparation of this antigen but a different
insert or the vector alone should be used as controls to confirm that the response to the TSH-R is protein specific. In addition, the development of T cell lines or T cell clones specific for a TSH-R antigenic preparation would further confirm that the T cells are specific for the TSH-R protein and would allow definition of the peptide epitope recognised. Furthermore, T cell line or T cell clone antigen presentation studies using T cells, derived from TAO patients, which have previously been incubated with an α-HLA class II antibody or an α-TCR antibody prior to assessing the proliferative response to a TSH-R antigenic preparation would elucidate the specificity of the response.
### Table 4.6. Counts per minute (cpm) and standard error of the mean (SEM) of EOM T cell lines and PBMC T cell lines from patient no.3 in response to thyroid membranes, EOM membranes, thyroid extract, EOM extract and the extracellular domain of the TSHR, tested in 2 separate experiments

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Concentration [μg/ml]</th>
<th>Counts per minute</th>
<th>SEM of cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly+F</td>
<td>10</td>
<td>131 cpm (21)*</td>
<td>705 cpm (154)*</td>
</tr>
<tr>
<td>Ly+F+PHA</td>
<td>947 cpm (53)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thy mem.</td>
<td>10</td>
<td>801 (30)</td>
<td>1040 (127)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>598 (109)</td>
<td>702 (56)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>398 (104)</td>
<td>959 (125)</td>
</tr>
<tr>
<td>EOM mem.</td>
<td>10</td>
<td>208 (67)</td>
<td>827 (113)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1144 (204)</td>
<td>1600 (55)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>605 (179)</td>
<td>1332 (485)</td>
</tr>
<tr>
<td>Thy extr.</td>
<td>10</td>
<td>2515 (157)</td>
<td>755 (57)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>435 (41)</td>
<td>815 (14)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>788 (62)</td>
<td>2844 (111)</td>
</tr>
<tr>
<td>EOM extr.</td>
<td>10</td>
<td>506 (101)</td>
<td>2120 (78)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>568 (28)</td>
<td>794 (211)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>466 (22)</td>
<td>751 (48)</td>
</tr>
<tr>
<td>TSHR-ECD</td>
<td>10</td>
<td>210 (31)</td>
<td>1713 (122)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>442 (55)</td>
<td>725 (6)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1118 (343)</td>
<td>748 (211)</td>
</tr>
</tbody>
</table>

Ly: lymphocytes; F: feeders; PHA: phytohaemagglutinin; NA: Not assayed; Thy: thyroid; mem.: membranes; EOM: extraocular muscle; extr.: extract, TSHR-ECD: extracellular domain of the thyrotropin receptor

*Figures in brackets refer to the SEM of the cpm of each triplicate sample.
4.3.5 EOM cytokine profile studied *ex vivo* by PCR

The presence or absence of transcripts for cytokines in 12 different EOM biopsies taken from a further five patients with TAO was also studied. A mixture of cytokine signals was detected: IL-1α, IL-2 and IL-10 mRNA were each found in 25% of the TAO biopsies, IL-4, IL-8 and TNF-α in 67% of samples, and IL-6 and IL-15 were found in 42% and 33% of biopsies, respectively (Fig. 4.1, page 182). IL-4 was detected in EOM biopsies from patient no. 10 in the absence of an amplification TCR product. This may represent mast cell- or fibroblast-derived cytokines $^{[577,578]}$. We were unable to amplify significant amounts of IL-1β, IL-12 (p40), IL-13 and IFN-γ from any of the twelve TAO extraocular muscle tissue cDNA samples (Fig. 4.1, page 182).
Table 4.7. Stimulation indices (SI) of EOM T cell lines and PBMC T cell lines from patient no.3 in response to thyroid membranes, EOM membranes, thyroid extract, EOM extract and the extracellular domain of the TSHR, tested in 2 separate experiments

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Conc. [µg/ml]</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EOM T cell line</td>
<td>PBMC T cell line</td>
<td>EOM T cell line</td>
<td>PBMC T cell line</td>
</tr>
<tr>
<td>Ly+F</td>
<td>1 (131 cpm)</td>
<td>1 (705 cpm)</td>
<td>1 (384 cpm)</td>
<td>1 (668 cpm)</td>
<td></td>
</tr>
<tr>
<td>Ly+F+PHA</td>
<td>7.2 (947 cpm)</td>
<td>2.3 (1623 cpm)</td>
<td>7.3 (2838 cpm)</td>
<td>13.4 (8978 cpm)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Thy mem.</td>
<td>10</td>
<td>6.1***</td>
<td>1.4</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.5 **</td>
<td>1.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.0 *</td>
<td>1.3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>EOM mem.</td>
<td>10</td>
<td>1.5</td>
<td>1.1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8.7 ***</td>
<td>2.2 *</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4.6 *</td>
<td>1.8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Thy extr.</td>
<td>10</td>
<td>19.0 ***</td>
<td>1.0</td>
<td>5.0***</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.3 **</td>
<td>1.0</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4.0 ***</td>
<td>4.0 **</td>
<td>1.3</td>
<td>4.2 ***</td>
</tr>
<tr>
<td>EOM extr.</td>
<td>10</td>
<td>3.8 *</td>
<td>3.0*</td>
<td>1.6</td>
<td>2.9 *</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.3 **</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.5 ***</td>
<td>1.0</td>
<td>2.2 *</td>
<td>1.0</td>
</tr>
<tr>
<td>TSHR-ECD</td>
<td>10</td>
<td>1.6</td>
<td>1.6</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.3 **</td>
<td>1.0</td>
<td>4.2 **</td>
<td>2.8 **</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>8.5 *</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Ly: lymphocytes; F: feeders; PHA: phytohaemagglutinin; NA: Not assayed; Thy: thyroid; mem.: membranes; EOM: extraocular muscle; extr.: extract, TSHR-ECD: extracellular domain of the thyrotropin receptor; *** (p<0.001) ** (p<0.01) * (0.02<p<0.06) Statistical analysis refers to comparisons between SIs from EOM- and PBMC-derived T cell lines using the same antigenic preparation.
Fig. 4.1. Cytokine gene expression in TAO EOM tissue using RT-PCR. For each oligonucleotide primer pair, cDNA template from all EOM specimens as well as that from the blood control were tested simultaneously. LMR: Left Medial Rectus  LIR: Left Inferior Rectus  RMR: Right Medial Rectus  RIR: Right Inferior Rectus. [Analysis kindly performed by Dr. R. A. Ajjan at the Department of Medicine, University of Sheffield, UK.]
Fig. 4.2. Cytokine gene expression in strabismus (non-TAO) control EOM tissue using RT-PCR. For each oligonucleotide primer pair, cDNA template from all EOM specimens as well as that from the blood control were tested simultaneously. LMR: Left Medial Rectus  LLR: Left Lateral Rectus  LIR: Left Inferior Rectus  RMR: Right Medial Rectus. [Analysis kindly performed by Dr. R. A. Ajjan at the Department of Medicine, University of Sheffield, UK.]
Cytokine transcripts were only identified for IL-6 and IL-8 in two of the five EOM biopsies from the three strabismus non-TAO control patients studied. These results are shown in Fig. 4.2 (previous page). TCR mRNA was amplified in 10 of the 12 TAO biopsies studied, and in all five biopsies from control patients, confirming the presence of T cells in almost all biopsies. In the controls, this presumably relates to a blood-borne population of cells. Control reactions with primers for β-actin were all positive, and those without cDNA remained consistently negative. Additional controls, in which reverse transcription was omitted, were always negative.

4.4 DISCUSSION

The present chapter concerns autoreactive T cells derived solely from EOM biopsies harvested from patients with TAO. EOM-derived T cell lines were mainly CD4+, in agreement with the immunohistochemical results from frozen EOM biopsies, in which the ratio of CD4+:CD8+ infiltrating T cells was at least 2:1 [290]. Our predominantly CD4+ T cell lines from the EOMs contrast with the predominantly CD8+ phenotypic profiles reported recently on T cell lines (cultured in IL-2 and further stimulated with anti-CD3 antibody) [393] and T cell clones (produced in IL-2-conditioned medium and stimulated with PHA) [392] which were derived from orbital fat/connective tissue specimens obtained in the course of decompression surgery. As expected, these T cell lines were CD25+ and HLA-DR+, although there was some variability between the lines. The difference in the origin of the biopsy material might explain these discrepancies. A more recent study, however, found predominantly CD4+ expression in T cell lines generated from retro-ocular connective tissue using IL-2 and anti-CD3 MoAb to expand the lines [403].

Functionally, our EOM-derived T cell lines generally released a mixture of
IL-4, IFN-γ, IL-10, TNF-β, TGF-β1, and IL-2, with one or another cytokine being predominant, particularly IFN-γ. Nevertheless, none released IFN-γ or IL-4 alone, which would typify a Th1 or Th2 subtype response. In addition, none of the lines produced detectable levels of IL-12 at any of the time points after stimulation (12, 24, 48 h). A problem with this analysis is how representative the lines are of the in vivo situation. To address this we looked at a further set of biopsies from TAO patients using RT-PCR to detect cytokine mRNA.

In vivo studies of the cytokine profile in TAO have been limited owing mainly to difficulties in obtaining tissue samples, in addition to the technical problems related to small specimen size and the patchy nature of the disease. The high sensitivity and specificity of RT-PCR enabled us to investigate the cytokine mRNA profile in small TAO tissue samples (1-2 mg). This technique, however, has its limitations; it is difficult to quantify and, in common with other mRNA based techniques, the results may not correlate with protein secretion. Only one other study to date has analysed the cytokine profile in TAO using a single EOM sample, and a Th2-like response was identified\(^{396}\). In the same study, a Th-2 like response was also detected in two out of the five orbital fat/connective tissue TAO samples studied\(^{396}\).

This is supported by the current work, where a large number of EOM specimens and an extended range of cytokines were assessed, in at least eight specimens from four TAO patients, by IL-4 mRNA expression together with a total lack of IFN-γ mRNA expression. Moreover, IL-6 mRNA detection in half the samples and IL-10 mRNA expression in a minority further suggest a predominantly Th2-like immune response in TAO. Expression libraries have shown that
immunoglobulin V genes can be detected in EOMs \cite{579}, and this cytokine pattern suggests that any sequences so derived may have pathological relevance and adds strength to the hypothesis that autoantibody synthesis can occur within the EOMs. Although the RT-PCR results may be more representative of what is happening \textit{in vivo}, it is also possible that because of the patchy inflammation, especially in later stages of the disease, biopsies might have been taken from an area not infiltrated by activated T cells. This could account for the variability between muscles from the same patient.

Most samples were positive for IL-8 mRNA expression, suggesting a role for IL-8 in mediating lymphocytic infiltration in TAO \cite{580}. The ability of TNF-\(\alpha\) to induce GAG synthesis by and proliferation of fibroblasts \cite{581} and its detection in more than half the samples also suggest the involvement of this cytokine in the pathogenesis of the disease. Thus, production of such a range of cytokines might be responsible for the anatomic site-selective alterations in fibroblast activation, altered cellular morphology \cite{417}, proliferation and changes in collagen synthesis \cite{187}, as well as GAG accumulation \cite{423}.

It is important to stress that the cytokines and products released by EOM-derived T cell lines may simply reflect their PHA-driven expansion \textit{in vitro}. More specifically, the high frequency of IFN-\(\gamma\) and IL-2 production by the biopsy-derived T cell lines could reflect PHA expansion of a few activated cells. The original cell number was too small (\(\approx\) 30 cells) to allow useful information to be derived without PHA plus IL-2 expansion. Given the differences between the starting material and the expanded T cell lines, it is perhaps not surprising that there is no direct correlation between the ELISA and PCR cytokine data in this study. The combined
results from both techniques at least permit an estimate of the potential for cytokine production within the EOMs in TAO.

It has been observed previously that the involved EOMs in TAO have often a patchy lymphocytic infiltration with a predilection for inferior and medial recti muscles. One of the inferior rectus biopsies studied, (patient no. 3), provided the highest yield of T cells and the most vigorously growing T cell line. This IL-2 dependent T cell line produced high levels of IL-4, IFN-γ and IL-10 in comparison to the levels obtained from other biopsies. In addition, sufficient numbers of T cells were generated from this biopsy to allow a study of antigen reactivity using thyroid gland and EOM extracts as well as a recombinant TSHR preparation. The proliferation data from this T cell line suggest that there are thyroid antigen- and TSHR-reactive T cells present in the EOMs, and in future experiments it will be important to determine the cytokine profile of the responding T cells. In many ways the TSHR is a logical candidate, since TAO is typically associated with Graves’ disease rather than autoimmune thyroid destruction. Arguments against this include the poor correlation between the severity of TAO and titres of thyroid-stimulating antibodies or TSH-binding inhibiting immunoglobulins. A second problem relates to the lack of convincing demonstration of a TSHR related protein in the orbit [197]. However, the present results support the hypothesis that TSHR-specific T cells may accumulate in the EOMs and at least contribute to the pathogenesis of TAO. Further studies, using expanded T cell lines and clones from the EOMs of TAO patients, are required to determine the frequency of TSHR-responsive T cells in TAO and their cytokine profile.
Chapter 5

An ultrastructural and systemic analysis of glycosaminoglycans (GAGs) in thyroid-associated ophthalmopathy
**5.1 INTRODUCTION**

Connective tissue GAGs are synthesized by the fibroblasts, and classified as chondroitins, keratans, dermatans, which are sulphated, and hyaluronan (or hyaluronic acid, HA), which is not \(^{[540]}\). A similar group, the heparans, are particularly associated with cell and basement membranes. All GAGs, except keratan sulphate, contain hexuronic acid as part of their molecule in a repeat disaccharide sequence with a hexosamine. In keratan sulphate the hexuronic acid is replaced by galactose. Owing to their polyanionic charge, GAGs osmotically attract and bind large amounts of water playing an important role in maintaining the tissue shape as well as in the formation of oedema during inflammation. Most of the signs and symptoms of TAO are explicable by the enlargement of the EOMs and the retro-ocular fat/connective tissue within the confined cavity of the orbit. GAGs are likely to play a major role in this process by increasing the volume of the affected tissues.

After secretion in the tissues, GAGs escape from the intercellular matrix into the circulation, are metabolised by the liver and finally excreted via the kidneys. The level and composition of GAGs present in serum and urine may therefore provide easily accessible indicators of the presence or activity of TAO. Kahaly *et al.* \(^{[559]}\) have demonstrated a possible increase in urine and plasma \(^{[582]}\) GAG levels in early TAO but another study \(^{[560]}\) of serum hyaluronan levels has shown no detectable change in the patients studied. To provide further information on the presence and localisation of GAGs in TAO we have taken biopsies of affected EOMs from TAO as well as non-TAO strabismus control patients and examined them by means of transmission electron microscopy (TEM) and immunogold staining using an anti-hyaluronan antibody. Serum hyaluronan was measured in TAO and control patients.
by a radioimmunoassay. Urinary GAG levels were assessed by photometric quantitation of hexuronic acids and the excretion pattern of the urinary GAGs of the same samples was determined by means of discontinuous electrophoresis with and without previous digestion with chondroitinase ABC.

5.2 PATIENTS AND METHODS

5.2.1 Ultrastructural immunohistochemistry

(1a) Extraocular muscle biopsies. Thirty-one biopsies of human EOM were collected from 18 TAO patients (6 males, 12 females; age 31-68 years), with their informed consent and Ethical Committee approval, during the course of corrective strabismus surgery where tight extraocular muscles were restricting normal ocular movements. Also 10 EOM biopsies were taken from 8 non-TAO strabismus control subjects (3 males, 5 females; age 12-55 years). TAO patients had clinically inactive disease for at least 6 months before the biopsies were collected and had not received any immunosuppressive therapy during this period of time. All biopsies were obtained from the belly of the muscle (10-12 mm from the insertion) having dissected off the muscle sheath and without prior application of any diathermy.

(1b) Transmission electron microscopy (TEM). For morphological investigation specimens were fixed in either a mixture of 2.5% glutaraldehyde and 0.5% tannic acid in 0.1M sodium cacodylate, pH 7.0 or 3% glutaraldehyde and 1% paraformaldehyde in 0.1M sodium cacodylate adjusted to pH 7.4 with 0.1M HCl. Samples were osmicated for 2 hours at 4 °C in 1% aqueous osmium tetroxide, dehydrated through ascending alcohols (50 - 100%) and propylene oxide, and embedded in Araldite resin cured at 60 °C. Semi-thin (1μ) sections were cut and
stained with 1% alcoholic toluidine blue for examination by light microscopy and ultrathin (70nm) sections of selected areas contrasted by sequential staining with 1% (w/v) uranyl acetate in solution of 50% ethanol/water and Reynold's lead citrate and examined in a JEOL 1010 TEM at 80kV by two independent researchers.

(1c) Tissue fixation for immunogold staining. Specimens for immunolabelling were fixed in 2% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.1M sodium phosphate buffer for 1 hour [583], and then embedded in Lowicryl HM20 by the progressive lowering of temperature (PLT) method using a LEICA AFS unit (Leica, Milton Keynes, U.K.). On two occasions specimens were quick frozen in a liquid nitrogen slush (-212 °C), freeze substituted with 10 changes of dried methanol at -80 °C, and infiltrated with Lowicryl HM20 at 50 °C again using the AFS unit. Lowicryl HM20 was polymerised by UV irradiation at -50 °C within the AFS unit. Ultrathin sections were collected on formvar/carbon coated grids for on grid labelling.

(1d) Immunogold staining. The buffer used throughout the procedure was 50mM Tris-HCl (pH 7.4) with 0.5M NaCl. Ultrathin Lowicryl sections mounted on formvar/carbon coated nickel grids were blocked by overnight incubation at 4 °C with 1% bovine serum albumin (BSA) in 50mM Tris-HCl buffer (pH 7.4). This was followed by a 4 hr incubation with the anti-hyaluronic acid antibody (raised in mice against human trophoblast membrane /Serotec, Oxford, UK) at a dilution of 1:10 at room temperature. After six 4 min changes in buffer to remove unbound primary antibody, the grids were preincubated with 1% BSA (5 min) and then incubated, for a further 1 hr, with a 1:50 dilution of a secondary goat anti-mouse 10 nm gold conjugate (Auroprobe, EM GAM IgM G10, Amersham, Aylesbury, UK). Unbound
gold conjugate was removed by three 4 min changes in Tris-HCl buffer, plus 0.2% BSA, followed by three changes in Tris-HCl buffer and washed in ultrapure distilled water. Finally, sections were stained sequentially with saturated uranyl acetate and Reynold’s lead citrate before being examined in a JEOL 1010 transmission electron microscope at 80kV by two independent researchers.

Two types of negative controls were incorporated into the immunogold procedure: (1) omission of the primary antibody and (2) substitution of the primary antibody with non-immune serum from the same species in which the primary was raised, both at dilutions identical to those used for the primary antibody.

### 5.2.2 Radiometric assessment of serum hyaluronan

Blood samples were obtained from 10 TAO patients (5 males, 5 females; aged 33-60 years, mean age 48), and 10 control subjects (5 males, 5 females; aged 35-60 years, mean age 48). Their clinical data are summarised in Table 5.1 (page 205). Blood samples were collected by venepuncture into sterile, glass vacutainer tubes with no additive and the serum separated by centrifugation (10 min, 400 g). Subsequently, the serum was aliquoted into 1 ml aliquots and stored at -40 °C until analysed.

The hyaluronan levels of these samples were measured radiometrically, according to the manufacturer’s instructions, using the Pharmacia HA kit (Pharmacia, Uppsala, Sweden) \(^{[584]}\). In principle, specific hyaluronan binding proteins (HABPs), isolated from bovine cartilage, which have a specific affinity for HA, are radiolabelled and used as a radiolabelled ligate. HA in the sample binds to \(^{125}\text{I}-\text{HABP}\) in a concentration dependent manner. Unbound \(^{125}\text{I}-\text{HABP}\) is then absorbed by HA immobilised onto Sepharose.
Each determination was performed in duplicate for both standards and unknown serum samples. A standard of known concentration provided by the kit supplier was serially diluted for each assay. The radioactivity of the standards and samples was measured in a γ-counter (Cobra Auto-Gamma, Model C5002, Canberra-Packard). The counts (B) for the standards and unknown serum samples were expressed as a percentage of the mean counts of the zero-standard (B₀) according to the formula: \[ \% \text{ activity bound} = \frac{100 \times B}{B_0} \]. Owing to the nature of the assay, the radioactivity counts inversely correlate to the amount of HA in the sample. The HA concentration was plotted against the percentage values of the standards and a standard curve was constructed. The concentration of the unknown serum samples was assessed using the following formula: \[ x = 4.15 - [0.038 \times y] \], which was found by fitting a linear curve into the lin-log plot of points of the standard curve using SPSS software, where \( x \) is the log of the HA concentration of the sample and \( y \) is the % of the activity bound.

### 5.2.3 Urinary GAGs

Twenty four hr urine collections were obtained from 37 different TAO patients (19 males, 18 females; aged 30-85 years / mean age 54 years), and 36 control subjects (17 males, 19 females; aged 24-69 years / mean age 47 years).

All reagents for this part of the study were purchased from Sigma, U.K, and were of the highest purity available, unless otherwise stated.
5.2.3.1 Quantitative estimation of urinary GAGs by measurement of hexuronic acid content

(1a) Isolation of GAGs. Initially, the undissolved constituents were centrifuged from 25 ml of the well mixed urine (10 mins; 400 g). One ml of the supernatant was used for the measurement of creatinine concentration. The hexuronic acid content of urinary GAGs was determined from another 1 ml aliquot by precipitation with cetyl pyridinium chloride (CPC) and ethanol \[^{[585,586]}\]. The pellets were dissolved in 200 µl of distilled water prior to assay.

(1b) Hexuronic acid assay. The aqueous GAGs were reacted with carbazole in the presence of borate/sulphuric acid according to the method of Bitter and Muir\[^{[587]}\] using γ-glucuronolactone standards. The concentration per litre was calculated from the standard curve. The value was five times the original urine concentration. Results were expressed in relation to the creatinine content of the urine samples, in order to take the urine concentration into account.

5.2.3.2 Identification of urinary GAG excretion pattern

Two 1 ml aliquots of the urine supernatant were precipitated in parallel, one of which was subsequently digested with chondroitinase ABC (Sigma C-2780, 100 mU/ml), as control. The enzyme digestion mixture was incubated for 1 hour at 37 °C. The residual GAGs in the enzyme-treated samples and the GAGs in the undigested samples were submitted to discontinuous electrophoresis.

(2a) Discontinuous electrophoresis. The presence or absence of chondroitin sulphate (CS), keratan sulphate (KS), dermatan sulphate (DS), and/or
heparan sulphate (HS) in 37 TAO and 36 control subject urine samples was studied. The constituent GAGs were identified using discontinuous electrophoresis as described by Hopwood and Harrison[588] with few modifications. Briefly, cellulose acetate plates (Titan III Zip Zone, 6.0 x 7.5 cm; Helena Laboratories Ltd., Newcastle, UK) were first immersed slowly and uniformly into cool (4 °C) 0.1M barium acetate (pH 5.0) in such a way that air was not trapped in the plates, and soaked for 20 mins. Subsequently, the sheet was blotted between filter papers (Whatman No 1 filter paper; BDH, Merck, Poole, UK). Meanwhile, 7 μls of either standard GAGs or urine sample were placed in each of the 8 wells of the sample trough. The applicator was primed by depressing 4 times the tips into the sample wells and this loading applied to a piece of filter paper. The plate was then placed in the aligning base, with the cellulose acetate side up, and its bottom edge aligned with the black scribe line “CENTER APPLICATION”. Following this, the samples were applied to the plate by depressing the applicator tips into the sample-well 5 times and promptly transferring the applicator to the aligning base. The button was pressed down and held for 6 seconds after which the plate was placed (cellulose acetate side down) in a cooling block in the electrophoresis chamber containing 0.1 M barium acetate (pH 5.0) and electrophoresed for 5 min at 200 V constant voltage. The electric current was switched off, the whole plate blotted with filter papers and then soaked in to cool (4 °C) 0.1 M barium acetate containing 15% ethanol for 2 min; it was again blotted and placed in the electrophoretic chamber. A 200 V constant voltage was applied for 30 mins. The plate was again removed from the chamber, blotted between filter papers and immersed for 2 min into cool (4 °C) 0.1 M barium acetate containing 50% ethanol. Having blotted the plate with filter papers a last electrophoretic step
was carried out with the same voltage conditions for 15 min. After the
electrophoresis the plate was stained in an aqueous solution (0.25%; w/v) of alcian
blue (Sigma, UK), destained in 1% acetic acid (BDH) rinsed under running tap
water, and dried by heated air.

A standard mixture of GAGs containing CS, KS, DS, and HS was
electrophoresed in a single lane and the identity of the GAGs in the unknown samples
determined by comparison with the mobilities of the standards. The quantity of each
GAG band was graded as (+++), (+), (±), and (−) relative to the intensity of the
staining of relevant standards graded as (+++).

5.2.4 Statistical analysis

Student’s t-test was carried out on the serum hyaluronan and urinary
hexuronic acid data with SPSS software and p value <0.05 was considered
significant in the comparisons between control and disease samples.

5.3 RESULTS

5.3.1 Ultrastructural immunohistochemistry

(1a) Extraocular muscle biopsies / Transmission electron microscopy

Transmission electron microscopy failed to reveal any significant differences
between the ultrastructural organisation of transversely sectioned extraocular muscle
fibres themselves in the TAO biopsies (Fig. 5.1, page199). However, in all TAO
specimens there was a marked expansion of the endomysial space in comparison to
the non-TAO biopsies (Fig. 5.2, page199). This space was filled by an increased
amount of collagen fibres interspersed with an amorphous material of low electron
density. Sections from tannic acid fixed biopsies also revealed in the same region an additional highly electron-dense material, most probably GAGs (Fig. 5.3, page 200). In addition, isolated myofibroblasts were seen in elevated numbers in the TAO specimens, as well as mast cells and fat cells in the endomysial space in TAO (Fig. 5.4, page 200). Perivenular inflammation was observed in TAO specimens (as shown previously [570]). The degree of inflammation correlated generally with the amount of collagen fibres and amorphous material detected in the extracellular matrix in between muscle fibres. Electron microscopy revealed evidence of both lymphocytes and monocytes within the inflammatory cuffs.
Fig. 5.1 Electron micrograph of Karnovsky-fixed EOM from a TAO patient. Note the presence of collagen fibres within the expanded endomysial compartment of the sample without concomitant disruption of individual muscle fibres. Scale bar = 10 μm, Mag. x 2400.

Fig. 5.2 Strabismus control EOM section prepared as in Fig. 5.1 showing much less collagen, no enlargement of the endomysium and morphologically normal muscle fibres. Scale bar = 10 μm, Mag. x 1600.
Fig. 5.3 Transverse section of a tannic acid fixed TAO EOM biopsy showing increased endomysial collagen deposits and electron dense GAG deposits (arrows) not seen in Karnovsky fixed samples. Scale bar = 5μm, Mag. x 4000.

Fig. 5.4 Selective enlargement of the abnormally expanded, collagen filled endomysial space from an EOM TAO biopsy showing a lipid droplet (L), a mast cell (big arrow), and diffuse amorphous material (small arrows). Karnovsky fixation. Scale bar = 1 μm, Mag. x 16 000.
Extraocular muscle biopsies / Immunogold staining. Post-embedding immunogold staining of ultrathin sections of Lowicryl embedded EOM biopsy material from TAO patients revealed positive staining for hyaluronan associated to the amorphous material surrounding striated collagen fibres (Fig. 5.5a) and (Fig. 5.5b, next page). Concomitant immunogold staining of ultrathin sections previously treated with hyaluronidase was not feasible owing to the small size of EOM biopsy material. Non-DAO strabismus control sections of EOM biopsies were negative (Fig. 5.6, next page).

Fig. 5.5a Ultrathin section of paraformaldehyde fixed, Lowicryl HM20 embedded EOM specimen from a TAO biopsy. Arrows show hyaluronic acid labelling of amorphous material. Scale bar =200 nm, Mag. x 65 000; .
Fig. 5.5b Ultrathin section of paraformaldehyde fixed, Lowicryl HM20 embedded EOM specimen from a TAO biopsy. Arrows show hyaluronic acid labelling of amorphous material. Scale bar =200 nm, Mag. x 100 000.

Fig. 5.6 Ultrathin section of a non-TAO, strabismus EOM biopsy prepared as in Fig. 5. There is not identifiable hyaluronic acid labelling. Scale bar = 200 nm, Mag. x 65 000.
Table 5.1. *TAO* and control patients' details and serum hyaluronic acid levels

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5.3.2 Radiometric assessment of serum hyaluronic

The HA concentration for each patient is shown in Table 5.1. In the 10 TAO patients studied, the mean serum concentration of HA was $21.8 \pm 17.2$ (SD) $\mu g/l$. This value is not statistically significantly different from that of the control subjects group whose mean HA concentration was $28.7 \pm 20.3$ (SD) $\mu g/l$ ($p > 0.5$; Student’s $t$-test).

5.3.3 Urinary GAGs

5.3.3.1 Quantitative estimation of urinary GAGs by measurement of hexuronic acid content

A statistically significant difference ($p = 0.03$) was found between the TAO patients ($6.08 \mu mol/l$ hexuronic acid/mmol/1 creatinine; SEM 0.456) and the control group ($4.8 \mu mol/l$ hexuronic acid/mmol/1 creatinine; SEM 0.283) in urinary GAG excretion. In the TAO patients studied hexuronic acid/creatinine ratio ranged from 2.0-11.8 $\mu$mol/mmol whereas in the control subjects studied it ranged between 2.7-10.0$\mu$mol/mmol.
### Table 5.2. Urine glycosaminoglycan analysis results in control specimens

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*Hex. Ac./Cr.: Hexuronic acid/Creatinine, C4S: chondroitin-4 sulphate, C6S: chondroitin-6 sulphate, KS: keratan sulphate, DS: dermatan sulphate, HS: heparan sulphate
† 2b: 2 bands
5.3.3.2 Identification of urinary GAG excretion pattern

Chondroitin sulphate was present in both TAO and control specimens although the frequency of appearance in the TAO samples as well as the intensity of the revealed bands was greater. No bands were detected in the aliquots treated with chondroitinase prior to discontinuous electrophoresis. Heparan sulphate was present in both TAO and control specimens with a pattern similar to that of chondroitin sulphate. Both control and TAO samples were mostly negative for keratan and dermatan sulphate. These results are summarised in Table 5.2 and Table 5.3 (next page)
Table 5.3. Urine glycosaminoglycan analysis results in early and late TAO specimens

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* Hex. Ac./Cr.: Hexuronic acid/Creatinine, C4S: chondroitin-4 sulphate, C6S: chondroitin-6 sulphate, KS: keratan sulphate, DS: dermatan sulphate, HS: heparan sulphate
† 2b: 2 bands
5.4 DISCUSSION

Previous electron microscopical studies of EOMs in TAO by Kroll and Kuwabara [204] have shown that the primary change in the EOMs is an interstitial inflammatory oedema and that alterations of muscle cells, when they occur, are probably a secondary phenomenon. This was confirmed a few years later by Riley [295]. Kroll and Kuwabara also noted that, in addition to inflammatory cells, fibroblasts were plentiful. Our morphologic data from transmission electron microscopy confirm previous findings by demonstrating a marked expansion of the endomysial space. We have now added to and expanded this earlier work by studying a larger number of TAO EOM biopsies and also by comparing them to control non-TAO strabismus specimens. In addition, in this study we have demonstrated that the enlargement of the endomysial space was caused by the presence of an increased amount of fibrous collagen, interspersed with a granular amorphous material surrounding striated collagen fibres. The amorphous granular material was proven by immunogold staining to have hyaluronan immunoreactivity in TAO not seen in control specimens.

Orbital fibroblasts obtained from TAO patients have been demonstrated to produce 2.9 times more HA in culture than those obtained from other non-TAO patients undergoing orbital surgery [589]. However, at the serum level, no statistically significant difference in the HA level was observed between the TAO and control subjects. The range of values obtained was similar in both groups, in agreement with the results of Imai et. al. [560] and the observations of Kahaly et. al. [582] who showed that in treated clinically inactive TAO patients normal plasma GAG values were found. A possible explanation for these results is that the amount of HA
overproduced locally might not be enough to influence the serum HA concentration, as has been shown in rheumatoid arthritis \cite{590,591}. Another possibility is that there could be an impaired removal mechanism for HA into the systemic circulation in TAO, owing to reduced venous drainage in the TAO orbits, and this might also be important in the accumulation of HA rather than solely enhanced production.

A statistically significant difference in the urinary GAG excretion was observed between the clinically inactive TAO and control patients studied, in agreement with previous findings for a similar group of patients \cite{558,559}. Specimens from active untreated TAO patients were not available to us for examination during this study. In addition, analysis of both TAO and control urine specimens by the qualitative technique of discontinuous electrophoresis, showed chondroitin sulphate and heparan sulphate to be present in both groups. However, the frequency of appearance in the TAO samples as well as the intensity of the revealed bands was greater. Treatment of the same samples with chondroitinase ABC resulted in the disappearance of the bands. This disappearance of the small GAG fragments produced by the enzymatic treatment may be due to either their increased solubility in the electrophoretic buffer or their failure to stain. The enzymatic loss of the heparan sulphate band may be owing to the observed digestion by either chondroitinase ABC or hyaluronidase to a small degree, even at room temperature, immediately after addition of the enzyme to the sample (personal observation, J.Stone). In only 3 of the male TAO and in none of the control specimens studied dermatan sulphate positive bands were observed. The significance of these bands is not known.

In conclusion, we have demonstrated by transmission electron microscopy
that there is consistently marked expansion of the endomysial space in TAO EOM biopsies as compared to non-TAO strabismus specimens. This expansion is caused by an increased number of collagen fibres, interspersed with a granular amorphous material surrounding striated collagen fibres shown to be hyaluronan by immunogold staining. Serum hyaluronan concentration was similar in the TAO and control specimens studied. In contrast, a statistically significant difference in the urinary GAG excretion was observed between TAO and control patients examined. However, discontinuous electrophoresis showed chondroitin sulphate and heparan sulphate to be present in both groups.
Chapter 6

CONCLUDING DISCUSSION
CONCLUDING DISCUSSION

In this thesis I have shown by means of immunohistochemistry that there is overexpression of vascular and cellular adhesion molecules in the interstitium and perimysium of EOM biopsies from patients with TAO correlating with early, active disease and this was reduced in the later stages of the disease \[592\]. In addition, also immunohistochemically, I have detected inflammatory cells (CD4\(^+\), CD45RO\(^+\), CD45RB\(^+\), and macrophages) as well as HLA class II enhanced expression in the interstitium of EOM biopsies from patients with early active disease. By contrast, in late TAO specimens, weaker HLA-DR expression and some macrophages only could be observed (submitted). Furthermore, I have isolated and expanded \textit{in vitro} the infiltrating T cells of affected EOM biopsies from TAO patients. Phenotypically, by flow cytometric analysis, the majority of the T cell lines were CD4\(^+\), CD45RO\(^+\), and TCR \(\alpha/\beta\). A wide variety of cytokines was detected by analysis of supernatants of these lines using ELISA techniques. In addition, cytokine mRNA present at the time of biopsy was determined by rapid extraction from EOMs and RT-PCR with specific oligonucleotide probes (IL-1\(\alpha\), IL-1\(\beta\), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IFN-\(\gamma\), TNF-\(\alpha\)), but the profiles were not identical comparing the two approaches. However, IL-4 was detected by both. Dose-dependent proliferation was observed in response to thyroid extract in a biopsy-derived T cell line suggesting that there are thyroid autoreactive T cells in affected EOMs in TAO \[561\]. Finally, I have determined the ultrastructural localisation of GAGs in the EOMs of patients with TAO and investigated if the quantity and type of GAGs present in blood and urine are markers of the disease, concluding that GAGs and in particular hyaluronic acid are present at the EOM level in patients with recently inactive TAO. However, serum
levels of hyaluronic acid and urinary GAGs are not sensitive indicators of their presence within the EOMs [593].

There are many questions we still cannot answer with respect to the pathogenesis of TAO [570] such as: (a) what is/(are) the antigen(s) shared by the orbit and the thyroid capable of driving the autoimmune reactions? (b) what is the target of T cells? (c) what is the mechanism whereby putative exacerbating factors such as cigarette smoking or radioiodine therapy affect or interplay with the immunological reactions taking place in the retro-ocular space? (d) why is the disease sometimes unilateral? (e) why do some TAO patients run into major, vision-threatening problems and others do not?

Nonetheless, the work described in this thesis along with other work in this area is able to provide a working hypothesis which provides several opportunities for immuno-intervention. This hypothesis proposes that autoreactive T cells directed against antigen(s) shared by the thyroid gland and the EOMs/retro-bulbar tissues are recruited by circulating or cell surface adhesion molecules and thereby infiltrate the retro-bulbar tissues and the perimysium of the EOMs. Here, activated T cells (and macrophages), after antigen recognition, secrete several cytokines that, in turn, stimulate fibroblasts to proliferate and secrete GAGs. The latter, in view of their hydrophilic nature, would ultimately be responsible for the clinical manifestations of the disease, including periorbital swelling, proptosis and increased EOM volume.

It is important to appreciate that unlike many chronic autoimmune inflammatory diseases, such as rheumatoid arthritis, the natural history of TAO appears consistent with a relatively short (e.g. two years) acute inflammatory phase. Furthermore, it has been argued that combined orbital radiotherapy and short term (e.g. six months) medical immunosuppression (with azathioprine and low dose...
prednisolone) can be far more effective than either treatment alone in the management of active TAO, reducing the requirement for orbital decompression and strabismus surgery more than 4-fold \cite{594}. The current hypothesis on the pathogenesis of TAO offers unique opportunities for immuno-intervention. Although chronic therapy would be impractical, short term treatment with significant improvement of outcome becomes a possibility in TAO.

In view of the current work and other studies reviewed above, possibilities for immuno-intervention include anti-cytokine therapy and anti-adhesion molecule therapy. In all this work it is important to monitor disease activity and the current studies support the idea that disease activity may be “monitorable” non-invasively by measuring serum or urine GAGs although the current techniques will have to be significantly more sensitive.

In 1996, Bahn’s group showed that IL-1 receptor antagonist (IL-1ra) and soluble IL-1 receptor (sIL-1R) are potent inhibitors of IL-1-induced GAG production by cultured human retro-ocular fibroblasts \cite{595}. They suggested that these two compounds, shown in early trials to be safe when administered parenterally, may be useful in the prevention or treatment of TAO. The cytokine network is extremely complex, and a number of cytokines (and growth factors), in addition to IL-1, probably contribute to generating and perpetuating the changes that occur in the EOMs and the retro-ocular fat/connective tissue of patients with TAO. The relative contribution of IL-1 to these changes is undefined, and the question of whether neutralisation of this cytokine is sufficient to interrupt the cascade of events remains to be clarified. Indeed, it cannot be ruled out that other cytokines play a pivotal role in the pathogenesis of the disease. Accordingly, treatment with IL-1 antagonists, even if effective in inhibiting IL-1, may not necessarily modulate the action of other...
cytokines and produce important effects on the manifestation of TAO\textsuperscript{[596]}. Another attractive target for cytokine intervention would be tumour necrosis factor since techniques have already been developed for this\textsuperscript{[597]}. TNF is present in EOM and retro-ocular fat/connective tissue biopsies from TAO patients and is known to stimulate GAG production.

However, in order to design efficient immunotherapeutic approaches to this disease, other molecules involved in the cellular processes, such as Fas and Fas ligand, will need to be identified. The role of chemokines in attracting cells into tissues should be examined in TAO as this could open up a whole array of potential pathways which could be blocked. Alternatively, the ability to alter the profiles of cytokines produced by the infiltrating T cells is an attractive approach now that the T cells have been isolated from EOMs and retro-ocular fat/connective tissue. Clearly, the cytokine environment in which the T cells are to be found within the EOMs and/or the retro-ocular fat/connective tissue will play a crucial role in defining T cell function. Further studies determining which of the detected cytokines are “dominant” in the pathogenesis of TAO, for the different stages of the disease, would provide important clues as to which cytokines could be the targets of future therapies. This could be achieved by culturing dissociated cells from TAO affected tissues \textit{in vitro} without any extrinsic stimuli and assessing the amount of different cytokines produced over a prolonged period of time, far longer than that detected using any combination of mitogenic stimuli, by using the currently available binding assays of high specificity. In addition, it would be important to determine whether bioactive cytokine proteins are chronically expressed in disease affected tissues supporting the concept that their regulation at the pathological site is different from that in normal tissues. Furthermore, since cytokine expression alone is not informative unless the
relevant receptors are expressed, cytokine receptor expression would be informative to be monitored in TAO affected tissues either qualitatively *in situ* by immunohistology, or quantitatively in dissociated cells by flow cytometry.

As more forms of immunotherapy are developed further studies will be required to determine whether these are potentially applicable in TAO and clinical studies will be required to establish the safety, the effectiveness, and the favourable cost to benefit ratio of these agents. TAO remains the most difficult part of autoimmune thyroid disease to treat, and is an important continuing challenge for immunotherapy.

Nevertheless, it should be emphasized that the immunotherapeutic approach does not address the basic mechanism of the disease and that a fully satisfactory therapy for TAO probably requires a specific therapeutic measure acting on its aetiological factors. Autoreactive T and B cells preferentially accumulate within the target organ in any autoimmune process. The EOMs in TAO are thus likely to be a rich source of retro-ocular autoantibodies, after expression in phage display systems which allow multiple rounds of screening (e.g on retro-ocular fibroblasts) to enrich for sequences of interest \(^{598}\). The feasibility of this strategy has already been demonstrated, as IgG and IgA mRNA have been detected by RT-PCR in the EOM and retro-ocular fat/connective tissue in TAO \(^{579}\). The power of this approach lies in the selective antibody enrichment, conferred both by the disease process and by the rounds of screening, and also the ability to analyse huge numbers of antibodies. Perhaps by a combination of such strategies, the TAO enigma could finally be solved.
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*Anastasia Pappa*  
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APPENDIX 1

PUBLICATIONS AND PRESENTATIONS BY THE AUTHOR

1. **Pappa A.,** Fells P., Lightman S.

2. **Pappa A.,** Fells P., Lightman S.

3. **Pappa A.,** Sheraidah G., Fells P., Lightman S.

Structural Studies of the Extraocular Muscles of Patients with Thyroid-Associated Ophthalmopathy, Invest. Ophthalmol Vis Sci 1993; 34 No 4; 2056.

5. **Pappa A.,** Jackson P., Munro P.M.G., Fells P., Lightman S.

6. **Calder V & Pappa A.**

7. **Fells P., Kousoulides L., Pappa A.,** Munro P., Lawson J.

8. **Pappa A.,** Munro P.M.G., Fells P., Lightman S.
The Ultrastructural Immunohistochemistry of Thyroid-Associated Ophthalmopathy. ISA and AAPO & S joint meeting. Vancouver, B.C., Canada, June 1994; P 22.

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9. **Pappa A., Munro P.M.G., Howes R.C., Fells P., Lightman S.**
Book chapter "The Ultrastructural Immunohistochemistry of Thyroid-Associated Ophthalmopathy".

10. **Pappa A.** Eye muscle derived T-cell lines. Progress in Thyroid-Associated Ophthalmopathy; *A Satellite to the 11th International Thyroid Congress*; Sept. 1995. Guest Faculty - Invited speaker.

Cytokine Gene Expression in Extraocular Muscles from Patients with Thyroid-Associated Ophthalmopathy. J Endocrinol 1996; 151; P 102.

12. **Pappa A., Calder V., Fells P., Lightman S.**


15. **Pappa A., Jackson P., Stone J., Munro P., Fells P., Pennock C., Lightman S.**
From the poem "Ithaka"

Ithaka gave you the marvellous journey.
Without her you would not have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka would not have fooled you.
Wise as you have become, so full of experience,
You would have understood by then what these Ithakas mean.

- Kavafis, 1911

* Ithaka = the "goal"