CARBOHYDRATES IN
CHRONIC INFLAMMATORY DISEASE
A morphological study of N-acetylg glucosamine,
hyaluronan and chondroitin sul phates
in rheumatoid arthritis

A THESIS SUBMITTED TO THE UNIVERSITY OF LONDON
FOR THE DEGREE OF DOCTOR OF MEDICINE

BY

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Abstract

The presence in rheumatoid arthritis of abnormally increased terminal N-acetylglucosamine (GlcNAc) on serum immunoglobulin G and of serum antibodies to GlcNAc-containing carbohydrates suggest a special role for GlcNAc in this disease. My hypothesis was that GlcNAc provides a specific driving force to the disease process in rheumatoid arthritis. The initial aim was to investigate histochemically the expression of terminal GlcNAc and the GlcNAc-containing carbohydrate, hyaluronan, in synovium. Terminal GlcNAc was identified using a specific monoclonal antibody, Mab GN7. A method was developed for localising hyaluronan in synovium using the hyaluronan-specific binding region of cartilage proteoglycan monomer. For both carbohydrates, greatly increased cellular and extracellular expression was found in rheumatoid synovium compared to normal. The appearances were, however, non-specific, being also present in osteoarthritic synovium and in a range of inflamed non-synovial tissues. Interest in the extracellular matrix led to a comparative morphological study of hyaluronan and the related glycosaminoglycans, chondroitin-4-sulphate/dermatan sulphate and chondroitin-6-sulphate, and of the cell-surface hyaluronan receptor, CD44. Each glycosaminoglycan showed a distinctive distribution in the matrix of synovium, skin and skeletal muscle, suggesting different roles in tissue matrix function. Normal synovial intima, for example, was found to contain more hyaluronan than subintima and to carry a discrete band of chondroitin-6-sulphate. Each showed profound alteration, indicating matrix disorganisation, in inflammatory disease. CD44-positive cytoplasmic processes were present on fibroblasts in hyaluronan-rich areas; they extended through the matrix and appeared to link with processes from distant cells. This study does not support a specific role for GlcNAc and GlcNAc-containing carbohydrates in rheumatoid arthritis but shows that carbohydrates, particularly those of the matrix, contribute to the marked heterogeneity of tissue structure and its modification in inflammatory disease.
ACKNOWLEDGMENTS

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1.3.2.1. The role of the oligosaccharide moiety of glycosylated molecules 36

1.3.2.2. N-acetylglucosamine (GlcNAc) in rheumatoid arthritis - an example of the alteration of the carbohydrate moiety of a glycoprotein in inflammatory disease 38

1.3.3. Glycosaminoglycans 40

1.3.3.1. General considerations 40

1.3.3.2. Hyaluronan 41

1.3.3.3. Chondroitin sulphates 42

1.3.3.4. Alteration of glycosaminoglycans in disease 42

1.3.4. CD44 as a cell-surface receptor for hyaluronan and chondroitin sulphate 45

14. LOCALISATION OF CARBOHYDRATES IN TISSUE 46

14.1. General considerations 46

14.2. Localisation of oligosaccharides in synovium 47

14.3. Advances in the methods of localisation of glycosaminoglycans in tissue 47

2. MATERIALS AND METHODS

2.1. TISSUES AND CELLS 50

2.1.1. Tissue collection, processing and storage 50

2.1.2. Enzymatic disaggregation of rheumatoid synovium 51

2.1.3. Separation of monocytes from peripheral blood 51

2.1.4. Preparation of cytospins 52

2.2. PRIMARY REAGENTS 52

2.2.1. Antibodies 52

2.2.2. Lectins 54
2.2.3. Preparation of the binding region probe for hyaluronan

2.3. SECONDARY REAGENTS
2.3.1. Bridging antibodies
2.3.2. Conjugated antibodies
2.3.3. Streptavidin conjugates

2.4. HISTOCHEMICAL PROCEDURES
2.4.1. General considerations
2.4.2. Peroxidase developed with diaminobenzidine
2.4.3. Peroxidase developed with ethylaminocarbazole
2.4.4. Alkaline phosphatase-anti-alkaline phosphatase
2.4.5. Double staining with monoclonal antibodies using peroxidase and alkaline phosphatase-anti-alkaline phosphatase
2.4.6. Non-specific esterase activity
2.4.7. Double staining for non-specific esterase activity and a monoclonal antibody using alkaline phosphatase-anti-alkaline phosphatase
2.4.8. *Ulex europaeus* lectin-Type 1 staining using immunoperoxidase
2.4.9. Double staining with Ulex using immunoperoxidase and a monoclonal antibody using alkaline phosphatase-anti-alkaline phosphatase
2.4.10. *Bandeiraea simplicifolia* GS II (biotinylated) staining using peroxidase
2.4.11. Immunofluorescence visualisation of monoclonal antibody binding
2.4.12. Double immunofluorescence with monoclonal antibodies of differing class 62
2.4.13. Double immunofluorescence with monoclonal antibodies of same class 62
2.4.14. Binding region probe staining method using peroxidase 62
2.4.15. Binding region probe staining method using alkaline phosphatase 63
2.4.16. Binding region probe staining method using immunofluorescence 63
2.4.17. Hyaluronidase treatment of sections 63
2.4.18. Preparation of hyaluronan oligosaccharides 64
2.4.19. Binding region probe specificity controls 64
2.4.20. Double immunofluorescence with binding region probe and monoclonal antibody 64
2.4.21. Chondroitinase ABC and chondroitinase AC digestion of tissues for staining with monoclonal antibodies 2B6 and 3B3 65

2.5. MISCELLANEOUS 65
2.5.1. Dot-blotting 65
2.5.2. Biotinylation of reagents 66

2.6. MICROSCOPY 66
2.6.1. Light microscopy 66
2.6.2. Fluorescence microscopy 67
2.6.3. Photomicrography 67

7
3. RESULTS I: TISSUE EXPRESSION OF TERMINAL N-ACETYLGUCOSAMINE (GlcNAc) AND ITS ALTERATION IN INFLAMMATORY DISEASE

3.1. GENERAL INTRODUCTION

3.2. GENERAL CONSIDERATIONS OF TERMINAL GlcNAc EXPRESSION IN SYNOVIIUM

3.2.1. Introduction
3.2.2. Terminal GlcNAc expression in normal synovium
3.2.3. Terminal GlcNAc expression in rheumatoid synovium
3.2.4. Expression of terminal GlcNAc on synovial cell nuclei
3.2.5. Confirmation of specificity of monoclonal antibody GN7 for terminal GlcNAc
3.2.6. Conclusions of 3.2.

3.3. CYTOPLASMIC/MEMBRANE EXPRESSION OF TERMINAL GlcNAc IN RHEUMATOID SYNOVIIUM

3.3.1. Introduction
3.3.2. Markers of tissue macrophages
3.3.2.1. CD68 compared to non-specific esterase activity in rheumatoid synovium
3.3.2.2. CD68 compared to monoclonal antibody RFD7 in rheumatoid synovium
3.3.2.3. Summary and conclusions of 3.3.2.
3.3.3. Relationship of terminal GlcNAc to CD68
3.3.4. Dendritic cells
3.3.4.1. Relationship between monoclonal antibody RFD1 and CD68 in rheumatoid synovial tissue
3.3.4.2. Relationship between monoclonal antibody RFD1 and CD68 in cells disaggregated from rheumatoid synovium 81

3.3.4.3. Summary and conclusions of 3.3.4. 82

3.3.5. Relationship of terminal GlcNAc to RFD1 positivity 83

3.3.6. Conclusions of 3.3. 85

3.4. EXTRACELLULAR EXPRESSION OF TERMINAL GlcNAc IN RHEUMATOID SYNOVIMUM 86

3.4.1. Introduction 86

3.4.2. Distribution of extracellular terminal GlcNAc and its relation to structural features 86

3.4.3. Morphological relationship between extracellular terminal GlcNAc and glycosylated molecules present in the extracellular space 88

3.4.4. Conclusions of 3.4. 89

3.5. SPECIFICITY OF CHANGES IN TERMINAL GlcNAc EXPRESSION FOR RHEUMATOID SYNOVITIS 90

3.5.1. Introduction 90

3.5.2. Terminal GlcNAc expression in non-rheumatoid synovial disease 90

3.5.3. Terminal GlcNAc expression in inflammatory disease in non-synovial tissues 91

3.5.4. Conclusions of 3.5. 92

3.6. RELATION BETWEEN TERMINAL GlcNAc AND HYALURONAN 92

3.6.1. Introduction 92
3.6.2. Binding of monoclonal antibody GN7 to hyaluronan as assessed by dot blotting 92
3.6.3. Effect of pre-treatment of tissues with Streptomyces hyaluronidase on binding of monoclonal antibody GN7 93
3.6.4. Conclusions of 3.6. 94

3.7. OVERALL CONCLUSIONS OF CHAPTER 3 95

4. RESULTS II: MORPHOLOGICAL DISTRIBUTION OF THE GLYCOSAMINOGLYCANs, HYALURONAN, CHONDROITIN-4-SULPHATE/DERMATAN SULPHATE (Ch4S/DS) AND CHONDROITIN-6-SULPHATE (Ch6S) IN TISSUE AND ALTERATION IN INFLAMMATORY DISEASE 97

4.1. GENERAL INTRODUCTION 97

4.2. PROBLEMS AND DEVELOPMENTS IN ASSESSING THE MORPHOLOGICAL DISTRIBUTION OF HYALURONAN IN TISSUE 97
4.2.1. Introduction 97
4.2.2. Use of the binding region probe to identify hyaluronan in synovium 98
4.2.2.1. Cetylpyridinium chloride as fixative 98
4.2.2.2. Time course for incubation with the binding region probe 99
4.2.2.3. Fixatives for double labelling with monoclonal antibodies and the binding region probe 99
4.2.3. Confirmation of the specificity of the binding region probe for hyaluronan 100
4.2.4. Comparison of monoclonal antibody NDOG1 with the binding region probe for identifying hyaluronan 101
4.2.4.1. Comparison of staining of with monoclonal antibody NDOG1 and the binding region probe 101

4.2.4.2. Effect of Streptomyces hyaluronidase on staining with monoclonal antibody NDOG1 102

4.2.5. Conclusions of 4.2 104

4.3. DISTRIBUTION OF HYALURONAN IN SYNOVIIUM AND RELATION TO CELLULAR ELEMENTS 104

4.3.1. Introduction 104

4.3.2. Distribution of hyaluronan in normal synovium 104

4.3.3. Distribution of hyaluronan in diseased synovium 105

4.3.4. Relation of hyaluronan to macrophages and lymphocytes in rheumatoid synovium 108

4.3.5. Distribution of cell-associated hyaluronan in cells disaggregated from rheumatoid synovium 109

4.3.6. Conclusions of 4.3. 110

4.4. DISTRIBUTION OF CHONDROITIN-4-SULPHATE/DERMATAN SULPHATE (Ch4S/DS) AND CHONDROITIN-6-SULPHATE (Ch6S) IN SYNOVIAL TISSUE 110

4.4.1. Introduction 110

4.4.2. Distribution of Ch4S/DS and Ch6S in normal synovium 110

4.4.3. Distribution of Ch4S/DS and Ch6S in rheumatoid tissues 112

4.4.4. Distribution of Ch4S/DS and Ch6S in osteoarthritic synovium 114

4.4.5. Effects of chondroitinase ABC and chondroitinase AC 114

4.4.6. Conclusions of 4.4. 115
4.5. GLYCOSAMINOGLYCANS IN NON-SYNOVIAL TISSUES AND ALTERATION IN INFLAMMATORY DISEASE

4.5.1. Introduction

4.5.2. Distribution of hyaluronan, Ch4S/DS and Ch6S in tonsil

4.5.3. Distribution of hyaluronan, Ch4S/DS and Ch6S in normal skin and changes in inflammatory skin disease

4.5.4. Distribution of hyaluronan, Ch4S/DS and Ch6S in normal skeletal muscle and changes in inflammatory muscle disease

4.5.5. Conclusions of 4.5.

4.6. EXPRESSION OF THE EXTRACELLULAR MATRIX RECEPTOR CD44 IN TISSUE AND ALTERATION IN INFLAMMATORY DISEASE

4.6.1. Introduction

4.6.2. CD44 expression in normal and diseased synovium

4.6.3. CD44 in relation to hyaluronan in rheumatoid nodule

4.6.4. CD44 expression in normal and diseased skin

4.6.5. CD44 expression in normal and diseased skeletal muscle


4.7. OVERALL CONCLUSIONS OF CHAPTER 4

5. DISCUSSION

REFERENCES

APPENDIX I

APPENDIX II

APPENDIX III

APPENDIX IV

POSTSCRIPT
| Table 1.1. | Ultrastructural features distinguishing type A from type B synovial lining cells |
| Table 2.1. | Rabbit polyclonal antibodies used in this study |
| Table 2.2. | Monoclonal antibodies used in this study |
| Table 2.3. | Secondary reagents used in this study |
| Table 3.1. | Patterns of terminal GlcNAc expression in rheumatoid synovium |
| Table 3.2. | The distribution in normal and rheumatoid synovial lining of CD68 in relation to non-specific esterase activity |
| Table 3.3. | The distribution in normal and rheumatoid synovial lining of CD68 in relation to RFD7 |
| Table 3.4. | The distribution in disaggregated rheumatoid synovial cells of terminal GlcNAc in relation to the macrophage marker CD68 |
| Table 3.5. | The mean (range) percentage of RFD1+ cells also CD68+ in different areas of rheumatoid synovium (n=9) |
| Table 3.6. | The distribution in disaggregated rheumatoid synovial cells of the dendritic cell marker RFD1 in relation to the macrophage marker CD68 |
| Table 3.7. | The distribution in disaggregated rheumatoid synovial cells of terminal GlcNAc in relation to the dendritic cell marker RFD1 |
| Table 3.8. | Extracellular terminal GlcNAc distribution in rheumatoid synovium in relation to structural features of the tissue |
| Table 3.9. | Codistribution of fibrin and extracellular terminal GlcNAc in rheumatoid synovium |
| Table 4.1. | Assessment of fixatives for suitability for double-labelling with the binding region probe and monoclonal antibodies |
| Table 4.2. | Effect of hyaluronidase treatment on staining of hyaluronan-rich tissues with Mab NDOG1 |
Table 4.3. Patient details and site of origin of synovium

Table 4.4. Distribution of chondroitin-4-sulphate/dermatan sulphate and chondroitin-6-sulphate in normal, rheumatoid and osteoarthritic synovium

Table 4.5. Morphological distribution of glycosaminoglycans in normal skin

Table 4.6. Hyaluronan, chondroitin-4-sulphate/dermatan sulphate and chondroitin-6-sulphate distribution in normal skeletal muscle
LIST OF FIGURES

Figure 1.1. Normal synovium
Figure 1.2. Rheumatoid synovium
Figure 3.1. Terminal GlcNAc expression in normal synovium
Figure 3.2. Terminal GlcNAc expression in rheumatoid synovium
Figure 3.3. Expression of terminal GlcNAc on synovial cell nuclei
Figure 3.4. Specificity of Mab GN7 for terminal GlcNAc compared to Bandeiraea
Figure 3.5. GlcNAc+ rheumatoid synovial cell with dendritic morphology
Figure 3.6. CD68 compared to non-specific esterase activity in rheumatoid synovium
Figure 3.7. CD68 compared to RFD7 positivity in rheumatoid synovium
Figure 3.8. Relationship of terminal GlcNAc to CD68 on rheumatoid synovial cells
Figure 3.9. Relationship between CD68 and RFD1 positivity in rheumatoid synovial cells
Figure 3.10. Relationship between terminal GlcNAc and RFD1 positivity in rheumatoid synovial cells
Figure 3.11. Distribution of extracellular terminal GlcNAc in rheumatoid synovium
Figure 3.12. Terminal GlcNAc expression in osteoarthritic synovium
Figure 3.13. Terminal GlcNAc expression in tuberculous abscess wall
Figure 3.14. Binding of Mab GN7 to hyaluronan as assessed by dot blotting
Figure 3.15. Effect of prior hyaluronidase digestion on Mab GN7 binding to rheumatoid synovium
Figure 4.1. Binding of the binding region probe for hyaluronan to rheumatoid synovium fixed with cetylpyridinium chloride
Figure 4.2. Binding region probe for hyaluronan compared to Mab NDOG1 on normal skin
Figure 4.3. Effect of hyaluronidase on binding of Mab NDOG1 to normal skin
Figure 4.4. Distribution of hyaluronan in normal synovium
Figure 4.5. Distribution of hyaluronan in diseased synovium
Figure 4.6. Relation of hyaluronan to macrophages and lymphocytes in rheumatoid synovium
Figure 4.7. Distribution of cell-associated hyaluronan in rheumatoid synovial cells
Figure 4.8. Distribution of Ch4S/DS and Ch6S in normal synovium
Figure 4.9. Distribution of Ch4S/DS and Ch6S in rheumatoid synovium
Figure 4.10. Distribution of Ch4S/DS and Ch6S in rheumatoid nodule
Figure 4.11. Distribution of Ch4S/DS and Ch6S in tonsil
Figure 4.12. Distribution of hyaluronan in normal skin
Figure 4.13. Distribution of Ch4S/DS and Ch6S in normal and psoriatic skin
Figure 4.14. Distribution of hyaluronan, Ch4S/DS and Ch6S in normal skeletal muscle
Figure 4.15. Distribution of hyaluronan, Ch4S/DS and Ch6S in polymyositisic muscle
Figure 4.16. Distribution of CD44 in normal and diseased synovium
Figure 4.17. Cellular distribution of CD44
Figure 4.18. Distribution of CD44 in relation to hyaluronan in rheumatoid nodule
Figure 4.19. CD44 in normal and psoriatic skin
Figure 4.20. CD44 in normal and polymyositisic muscle
1. INTRODUCTION

1.1. STRUCTURE OF NORMAL SYNOVİUM

1.1.1. Synovial joints and other synovial structures

The adult human skeleton contains joints of three types (Gardner E et al 1969): fibrous joints (synarthroses), fibro-cartilaginous joints (symphyses) and synovial joints (diarthroses).

Synovial joints are complex structures specialised to permit a wide range of movement with low frictional drag (McCutchen CW 1981). They are characterised by the presence of a cavity and specialised connective tissue, synovium. The joint space contains a small volume of synovial fluid, which owes its high viscosity to the presence of hyaluronan (Gardner E et al 1969). Hyaline cartilage covers the bone ends and the whole joint is enclosed in a capsule of fibrous tissue. Synovium is attached to bone at the junction with hyaline cartilage and is reflected off this junction to line the whole capsule. Normally, folds of synovium fill the spaces between non-congruous cartilage surfaces. These folds slip away during movement as the point of contact between opposing cartilage surfaces shifts (Henderson B et al 1987) but close apposition of the soft, deformable synovium and cartilage is maintained by sub-atmospheric intra-articular pressure (Reeves B 1966; Levick JR 1979). The smooth surface of hyaline cartilage, the lubricating qualities of synovial fluid and the deformability of synovium may all serve to minimise friction during joint movement.

Synovium is found at other sites where movement occurs between tissue planes, such as tendon sheaths and bursae (Canoso JJ 1981). Clearly, a disease for which synovium is the target tissue will affect all these sites.
1.1.2. General structure and function of synovium

Synovial structure is variable. Macroscopically, small villi are often seen on the synovial surface opposite the joint line while elsewhere the surface is smooth and flat. There is also wide variation in the microscopical structure. The synovial intima or lining layer may consist of a scattering of flattened cells through a spectrum to a dense band packed with cells several deep (Henderson B et al. 1987) (Figure 1.1.). The number of cells associated with vessels also varies markedly and synovium from normal joints may overlap in the apparent degree of cellular infiltration with synovium from joints which are clinically inflamed (Lindblad S et al. 1987). Deeper layers may contain fat or have the appearance of loose areolar or dense fibrous tissue (Reith EJ et al. 1970). Although it is known that synovium from different areas of the same joint may vary there has been no formal study in humans of the association between histological appearance and site within the joint.

Figure 1.1. Normal synovium. (Haematoxylin and eosin x 20)

Like all tissues, synovium is composed of cells embedded in matrix. In normal synovium, the volume ratio of interstitial matrix to cells is high (Figure 1.1.), in
contrast to inflamed synovium where the tissue often appears to be little more than a dense collection of cells loosely held together by strands of matrix (Figure 1.2.). The cells of normal synovium include fibroblasts, macrophages and a small number of mast cells and lymphocytes (Henderson B et al 1987; Lindblad S et al 1987). Dense vascular networks are present approximately 10 microns and 100 microns beneath the synovial surface (Wilkinson LS et al 1989) and lymphatics (Davies DV 1946) and free nerve endings (Dee R 1978) have been identified.

Figure 1.2. Rheumatoid synovium. (Haematoxylin and eosin x 20)

The functions of the synovial lining appear to be to act as a deformable packing material, to provide a smooth, frictionless, non-adherent surface to aid joint movement and to assist in cartilage nutrition (cartilage is avascular). Synovial lining cells may also produce components of synovial fluid, which may be important in lubrication. (Henderson B et al 1987)

Synovium, like articular cartilage, lacks a continuous, limiting cellular layer containing tight junctions and a basement membrane (Luse SA 1960). Although
synovial tissue matrix is therefore in some sense continuous with the synovial fluid, it nevertheless provides significant resistance to fluid flow. Indeed, the blood-joint space barrier has been described as series of three fibrous mats - the endothelial fenestral glycocalyx, the endothelial basement membrane and the synovial interstitial matrix - which determine tissue porosity and which may therefore play a role in regulating synovial fluid volume (Levick JR 1989). Synovial fluid is basically a capillary dialysate with the addition of hyaluronan in high concentration. It is generally assumed that synovial fluid hyaluronan originates from the synovial lining cells and is secreted directly into the joint cavity but this has yet to be established.

1.1.3. Synovial lining cells

Electron microscopy distinguishes two types of synovial lining cells (Barland P et al 1962), designated Type A and Type B cells respectively. The distinguishing features (Table 1.1.) suggest that Type A cells are capable of phagocytosis and a degree of synthetic activity and resemble macrophages whereas Type B cells are specialised mainly for protein synthetic activity and resemble fibroblasts.

Table 1.1.

ULTRASTRUCTURAL FEATURES DISTINGUISHING
TYPE A FROM TYPE B SYNOVIAL LINING CELLS

<table>
<thead>
<tr>
<th>TYPE A CELLS</th>
<th>TYPE B CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dense nuclear chromatin</td>
<td>Open nuclear chromatin</td>
</tr>
<tr>
<td>Nucleoli infrequently seen</td>
<td>Nucleoli prominent</td>
</tr>
<tr>
<td>Numerous vacuoles, vesicles</td>
<td>Few vesicles</td>
</tr>
<tr>
<td>Moderate amount of RER and scattered free ribosomes</td>
<td>Large amount of RER</td>
</tr>
<tr>
<td>Prominent Golgi</td>
<td>Less prominent Golgi</td>
</tr>
</tbody>
</table>

Key: RER = rough endoplasmic reticulum
(Adapted from Henderson B et al 1987)
Intermediate cells with the electron microscopic features of both Type A and Type B cells, although rare in normal synovium, are abundant in diseased tissue (Kinsella DT et al 1970) and gave rise to a debate regarding ontogeny. One early view (eg Kinsella DT et al 1970) was that the intermediate cells represented a single precursor cell residing in the synovium from which cells of both Type A and Type B might arise. A more recent view (eg Ghadially F 1983) held that the presence of intermediate cells suggested that Type A and Type B phenotypes were produced within a single cell by local environmental effects. However, activated cells of various types may show similar electron microscopic appearances and the cells of so-called intermediate morphology in synovium may be cells of both Type A and Type B which on activation have come to express similar features. Further evidence against the existence of an intermediate cell type is provided by work (Graabeck PM 1982) on ultrathin serial sections of normal rat synovium which demonstrated the presence of only two cell types; cells of intermediate appearance were invariably found to be Type B cells on further sectioning.

It is now generally accepted that the Type A cell is a bone-marrow derived macrophage (Edwards JCW et al 1982) while Type B cells are assumed to be of local origin. The availability of monoclonal antibodies (Mabs) to surface markers associated with macrophages has revealed marked heterogeneity among synovial macrophages; several studies have shown different markers to identify distinct, if overlapping, populations (Allard SA et al 1990; Hogg N et al 1985; Broker BM et al 1990). The recent discovery of a marker, CD68, which appears to be present on all cells of monocyte/macrophage lineage (Kelly PMA et al 1988) promises to aid the identification of the total macrophage population in synovium. No specific marker for fibroblasts has so far been discovered but the search for a marker of synovial fibroblasts, in particular, has been advanced by the recent development and characterisation of a Mab which apparently preferentially recognises a specific product of fibroblastic synovial lining cells (Stevens CR et al 1990).
How lining cells contribute to the functional properties of synovium is largely unknown as functional studies on the cells have been hampered by the difficulty of separating them from the tissue. Most studies of synovial cells have been performed on enzymatically disaggregated tissue or on cells grown out from explants; however, without prior dissection of the lining layer both techniques will clearly yield cells derived from the whole depth of the tissue, rather than the lining layer alone. The disaggregated cells are initially a heterogeneous mixture but after several passages in culture they take on a uniform fibroblastic morphology (Dayer J-M et al 1976). Cells grown from explants are uniformly fibroblastic. A method which seems more likely to yield lining cells alone involves limited trypsinisation of the synovial lining of the intact joint (Fraser JRE et al 1965) but in humans this method may yield largely synovial macrophages (Dahl IMS et al 1985). A problematic feature of cell culture is increasing deviation from the differentiated phenotype expressed in vivo with passaging (Georgescu HI et al 1988). As no specific feature or marker of the synovial fibroblast has yet been identified, for these cells there is no reliable measure of how closely a cultured cell resembles its in vivo counterpart.

Fibroblasts cultured using the above methods produce a characteristic pericellular halo of matrix (Clarris et al 1967) which contains hyaluronan and chondroitin sulphates as the major glycosaminoglycan constituents (Hamerman D et al 1982) and Type I and Type III as the major collagens (Vuorio E 1977). They synthesise matrix- degrading enzymes (Clarris BJ et al 1987; Dayer J-M et al 1976 ), and have shown themselves susceptible of stimulation by cytokines (McGuire-Goldring MB et al 1984; Yaron I et al 1989). But owing to the problems discussed above, it cannot be assumed that the specific fibroblastic cell of the synovial lining will exhibit similar behaviour.

Despite the lack of evidence, it has been assumed that it is the fibroblastic lining
cell which synthesises the hyaluronan and other molecules present in synovial fluid and which is in some way responsible for maintaining the smooth frictionless surface of synovium.

1.1.4. Extracellular matrix

1.1.4.1. General considerations

Tissue matrix, far from being the ‘amorphous ground substance’ of traditional histology (Reith EJ et al 1970), is now known to be a complex, highly organised structure (Hay ED 1991) with the following general functions:

(i) to provide a physical support for resident and visiting cells
(ii) to withstand mechanical stress
(iii) to permit/facilitate the passage of nutrients and chemical messengers and other secreted products
(iv) to maintain cell behaviour appropriate to the normal functioning of the tissue as a whole

Tissue extracellular matrix comprises the interstitial matrix and the basement membrane which is a thin layer, different in composition from the interstitium, immediately adjacent to the cell. In certain tissues, the basement membrane becomes organised into a specialised zone immediately underlying epithelial, mesothelial and endothelial tissues which line cavities (Reith EJ et al 1970).

Carbohydrates and carbohydrate-bearing proteins are the predominant constituents of the extracellular matrix. The matrix of all tissues contains a mixture of glycosaminoglycans and proteoglycans, the fibrous glycoproteins, collagen and elastin, and structural glycoproteins (Hay ED 1991), of which fibronectin and laminin have been the most extensively investigated. Proportions of the major constituents vary and this, together with the incorporation of specific minor constituents, gives rise to widely differing matrices with properties
appropriate to individual tissues. Tendon matrix, for example, comprises densely-packed, longitudinally-arranged fibres of Type I collagen separated by a fine meshwork of Type III collagen whose interstices are occupied by hydrated proteoglycan molecules (Canoso JJ 1981). This arrangement confers the high tensile strength and almost perfect elasticity necessary in a structure specialised for the transmission of muscle pull to bone. A very different matrix is that of articular cartilage which comprises large, under-hydrated proteoglycan aggregates constrained by a meshwork of Type II collagen fibres (Ghadially FN 1981). The swelling pressure of the proteoglycans confers the ability to resist compression essential in a load-bearing tissue.

Glycosaminoglycans and proteoglycans fill the interstices of the network formed by the fibrous proteins, conferring bulk and deformability and providing a water-based environment. They may also function as a repository for modulators of cell behaviour. Sulphated glycosaminoglycans bind growth factors and matrices of certain cultured cells have been shown to contain, bound as insoluble complexes, basic fibroblast growth factor (Vlodavsky I et al 1987; Rifkin DB et al 1989), granulocyte-macrophage colony stimulating factor (Gordon MY et al 1987) and interleukin-3 (Roberts R et al 1988). Interestingly, transforming growth factor-β1 binds to the core protein of the chondroitin sulphate/heparan sulphate-bearing proteoglycan, betaglycan, rather than to its glycosaminoglycan chains (Chiefetz S et al 1989).

Tissue matrix contains a wide variety of structural, non-collagenous glycoproteins, bound to its other elements. These may serve to stabilise the matrix and promote cell adhesion (Yamada KM 1992). The most extensively investigated matrix glycoprotein is fibronectin, which carries a number of binding sites for collagen, hyaluronan, fibrinogen, heparin and DNA and also two regions forming ligands for members of the integrin family of cell surface adhesion molecules (see
1.1.5). Fibronectin may play an important role in promoting cell growth, as has been shown most convincingly for endothelial cells (Ingber DE 1990). Unlike peptide growth factors, however, it appears to exert its effects only when presented in insoluble form, that is, organised in a complex matrix (or bound to tissue-culture plastic). Its growth-promoting effects appear to be related to its ability to stimulate cell spreading (Ingber DE 1990), exemplifying the interdependence of cell shape and cell behaviour.

1.1.4.2. Synovial extracellular matrix

Of the tissues of the joint, articular cartilage with its highly specialised matrix has been the most heavily investigated; comparatively little is known of the soft connective tissues such as synovium.

A range of collagens has been demonstrated in synovium by immunohistochemical studies. The main collagens present are Type I and Type III (Linc G et al 1983; Eyre DR et al 1975). Type VI collagen has recently been reported to be present within the superficial synovial lining layer (Levick JR et al 1990) and Types I, III, V and VI have been identified in rabbit synovium (Ashhurst DE et al 1991). Type IV collagen and laminin have been found in a pericellular distribution in the synovial lining (Pollock LE et al 1990). These are major constituents of vascular and epidermal basement membrane zones and the question arises as to whether they subserve similar functions in the synovial lining.

Fibronectin has been localised in rheumatoid synovium (see 1.2.5.) but there have been no studies on normal human synovium.

Studies of glycosaminoglycans and proteoglycans in whole synovial tissue have been limited to hyaluronan. The first attempt to localise hyaluronan in human synovium (Hamerman D et al 1959) used toluidine blue, which binds to negatively charged
molecules, in conjunction with streptococcal hyaluronidase digestion. Staining was present in the lining layer and abolished by enzymatic treatment. A second attempt (Roy S et al 1967) used a colloidal iron binding method and an electronmicroscopic technique in rabbit synovium. Iron particles were found in the matrix and on the surface of and within synovial cells and binding was abolished by treatment with bovine testicular hyaluronidase. However, both toluidine blue and colloidal iron bind to all glycosaminoglycans and both streptococcal and bovine testicular hyaluronidase degrade a number of these so it is questionable whether either method localises hyaluronan alone (see 1.4.3.).

The extracellular matrix constitutes the bulk of normal synovium, so it is presumably involved in the synovial functions of regulation of synovial fluid volume, maintenance of a smooth frictionless surface and the property of deformability. As little is known of its precise composition or ultrastructure, however, the nature of this involvement is unknown.

1.1.5. Cell-surface extracellular matrix receptors

The extracellular matrix clearly constitutes the most intimate environment of the cells embedded in it and interactions between cells and the surrounding matrix are important in determining cell behaviour. Cell-matrix interactions are involved in processes as diverse as the generation of epithelial cell polarity (Wang AZ et al 1990), neurite outgrowth (Lander AD 1990), platelet adhesion and aggregation (Andrews RK et al 1990) and tumour cell invasion (Knudson W et al 1989). Studies on limb development in the chick embryo have shown that changes in matrix composition mirror cellular changes (Craig FM 1988).

Cells interact with the matrix by means of surface receptors, the expression of which may be modulated by external stimuli (Hynes RO 1992). Interestingly, many of these receptors possess a number of ligands: the same molecules may
mediate cell-cell and cell-matrix adhesion and bind to multiple matrix constituents (Takada Y et al 1988; Springer TA 1990). The integrin family were first identified as cell-cell adhesion molecules but all members so far identified also show receptor activity for matrix substances such as collagen, fibronectin, laminin, vitronectin, fibronectin and thrombospondin (Springer TA 1990). Specificity of cellular response is, on present knowledge, difficult to explain but may theoretically be determined by differential receptor expression, simultaneous binding of ligand by several different receptors or by modification of ligand binding by alteration in the pattern of carbohydrate expression on adhesion molecules, may of which are known to be heavily glycosylated.

The most extensively investigated matrix receptor is the fibronectin receptor, integrin $\alpha_5\beta_1$. This molecule is expressed by fibroblasts, epithelial and endothelial cells, platelets and T lymphocytes (Springer TA 1990) and binds to an arginine-glycine-aspartate sequence on fibronectin (Ruoslhti E et al 1987). Ligand binding has been shown to stimulate cell spreading in endothelial cells (Ingber DE 1990) and expression of genes coding for degradative enzymes in fibroblasts (Werb Z et al 1989).

The principal receptor for hyaluronan is CD44 (see 1.3.4.), a multi-domain transmembrane glycoprotein which is not a member of any of the adhesion molecule families so far described. CD44 is present on a wide variety of cells but its distribution in synovium has not yet been described.

1.2. CHANGES IN SYNOVIAL STRUCTURE IN INFLAMMATION

1.2.1. The inflammatory process and changes in general tissue structure in inflammatory disease

Inflammation constitutes the response of living tissue to injury and serves to remove damaged tissue and initiate repair (Spector WG 1977). It is primarily
protective but in certain diseases the process appears to be amplified and prolonged and may result in irreversible tissue damage. Examples of such diseases are tuberculosis, where the cause is known, and Crohn's disease and rheumatoid arthritis, where the cause is unknown.

The first signs of inflammation are vasodilatation and exudation of proteinaceous fluid into the tissue, seen clinically as erythema and swelling respectively. Polymorphonuclear leucocytes (microphages) migrate through blood vessel walls into the tissue and they are followed by monocytes which rapidly mature into macrophages. These phagocytic cells remove debris and secrete vasodilator prostaglandins, chemotactic leukotrienes, cytokines and degradative enzymes. Endothelial cells are stimulated to form new blood vessels and tissue repair, by a combination of regeneration and fibrosis, is effected by activated fibroblasts.

If the inflammatory process is immune-mediated, lymphocytes infiltrate the tissue in large numbers (Roitt IM 1984). B lymphocytes mature into antibody-secreting plasma cells and T lymphocytes mature into cells specialised for cell-killing or cytokine secretion. Immune processes are triggered by antigen which is recognised, rightly or wrongly, as non-self. This antigen may be a chemical toxin or be present on the surface of a foreign cell, such as a bacterium. Indigenous cells may carry antigen that is recognised as abnormal if the cells are infected with virus or have undergone malignant change. In order for the immune process to be triggered, the antigen must be presented to the immune cells in a way they will recognise. Several cell types are able to present antigen but the specialised antigen-presenting cell or dendritic cell (whose lineage is controversial) is the most efficient (Steinman RM et al 1981).

If the primary insult persists, then chronic inflammation ensues. The affected tissue is disrupted: resident cells may be outnumbered many times over by
infiltrating cells, the matrix becomes disorganised and fibrotic and marked 
angiogenesis is present.

1.2.2. Rheumatoid arthritis as an example of inflammatory disease of synovium
The commonest chronic inflammatory synovial disease is rheumatoid arthritis
(Kelley WN et al 1989), the cause of which is unknown. The disease is systemic
and a variety of tissues may be affected but synovium forms the main target.
Synovium shows all the features of chronic inflammation but as yet no specific 
histological feature has been identified.

Probably the most specific clinical feature overall is the rheumatoid nodule which
is found in a subset of patients who have relatively severe disease, often associated
with vasculitis, and high titres of serum rheumatoid factor. Rheumatoid nodules are
firm, non-tender lobular masses typically found within the subcutaneous tissues
over bony points on the extensor surfaces of the limbs but occasionally in the
synovium, lung and heart. Histologically, they are characterised by microscopic
areas of necrosis surrounded by palisading macrophages but lymphocytes are
rarely seen (Bennett GA et al 1970; Fassbender HG 1975). Subcutaneous nodules
may occur in rheumatic fever (and were once thought to provide evidence of a
connection between rheumatic fever and rheumatoid arthritis) but such nodules
are evanescent and show differences in histological structure (Bennett GA et al
1970).

The lack of specific diagnostic features of rheumatoid arthritis has led to the
development of empirical criteria for use in research (see Appendix I), aimed at
identifying a relatively homogeneous group of subjects for study.

The cause of rheumatoid arthritis is unknown but, as in all disease, it appears that
a combination of environmental and genetic factors are relevant. The genetic link
is established in that certain genetically-determined human leucocyte-associated (HLA) antigens are found with greater frequency in patients with rheumatoid arthritis and their immediate relatives than in the normal population. The associations vary with race; in caucasoids, HLA DR4 is associated with a relative risk of 2.7 (Kelley WN et al 1989). Environmental factors are less well-established; the search for occult infection as a trigger has so far proved fruitless but continues with organisms as diverse as Epstein-Barr virus (Crawford DH et al 1983), mycobacteria (Rook GAW et al 1988) and Proteus mirabilis (Ebringer A et al 1985) as suspects.

As the disease attacks connective tissue preferentially, autoimmunity to connective tissue matrix components has been postulated as relevant to aetiopathogenesis. Experimental models show that Type II collagen, the predominant collagen in articular cartilage, can cause an inflammatory arthritis in rats and mice and the disease can be transmitted by transfer of the IgG fraction of serum, which contains anti-collagen antibodies (Stuart JM et al 1982), or by transfer of lymphocytes (Trentham DE et al 1978). Anti-collagen antibodies may arise in patients with rheumatoid arthritis and there has been at least one report of such antibodies from a patient causing an arthritis in mice (Wooley PH et al 1984), indicating pathogenicity. Recent work, however, has shown that these naturally-occurring anti-collagen antibodies react with denatured rather than native collagen (Rowley M et al 1986), suggesting that they are produced in response to tissue damage rather than acting as initiators. Nevertheless, they may serve to perpetuate the inflammatory process in joint tissues.

A possible role for autoimmunity to other matrix components, together with an infective aetiology, is suggested by the isolation of a T lymphocyte clone from rats with arthritis induced by immunisation with Mycobacterium tuberculosis which responded to cartilage proteoglycan and led to arthritis when injected into naive
animals (van Eden W et al 1985).

1.2.3. General synovial structure in rheumatoid arthritis

In rheumatoid arthritis the synovium loses its smooth, glistening pinkish-white appearance and instead becomes brownish (owing to the presence of haemosiderin derived from extravasated blood), friable and engorged. The surface is thrown into folds or villi which greatly increase the surface area presented to the joint cavity. Minor areas of synovial villous formation may be found in osteoarthritis and even in normal joints opposite the joint line but in rheumatoid arthritis the changes are gross, with masses of hypertrophied villi distending the joint capsule. Fibrin may be present on the synovial surface and within the substance of the tissue. Rice bodies, which are pearly white, smoothly-rounded elements found, often in large numbers, in the joint cavity, may be formed from shed fibrin and fragments of tissue debris.

Microscopically, the tissue is densely cellular, largely due to influx of cells from the vascular compartment rather than to local proliferation (Figure 1.2.). This is evidenced by the rarity of mitotic figures and the small number of cells marked by an antibody to a nuclear antigen expressed by proliferating cells (Lalor PA et al 1987; Henderson B et al 1985). Vascularity is greatly increased and high endothelial venules are present (Freemont AJ et al 1983), facilitating the diapodesis of lymphocytes.

Clearly, this mass of inflammatory synovial tissue cannot function as normal synovium and deterioration in joint function is seen early as painful restriction of the range of movement. Large volumes of synovial fluid are formed but the fluid is low in viscosity, owing to the reduced concentration of hyaluronan (Balazs EA et al 1967) and contains tissue debris, effete cells and inflammatory mediators (Kelley WN et al 1989); articular cartilage nutrition via synovial fluid is compromised.
In severe disease, the mass of inflammatory synovial tissue (known as “pannus” when it forms at the synovio-cartilage junction) encroaches on cartilage and bone, causing destruction of both tissues.

1.2.4. Synovial cell populations in rheumatoid arthritis

1.2.4.1. General considerations

The rheumatoid synovial surface is densely populated with cells, virtually all of which carry macrophage markers (Hogg N et al 1985), in contrast to normal synovium, where lining cells are predominantly fibroblastic. These macrophages express markers of activation, such as Class II (Hogg N et al 1985). Whether synovial fibroblasts also express Class II antigens in vivo is in dispute (Lindblad S et al 1983; Hogg N et al 1985) but certainly such expression can be induced on cultured fibroblasts by exposure to cytokines (Geppert TD et al 1987).

Macrophages are distributed throughout the deeper layers and especially around blood vessels, suggesting recent egress from the circulation. Small lymphocytes and plasma cells are rarely found within the lining layer but large numbers may be present in the deeper tissue, often in aggregates or even organised into follicles, although germinal centres are rare (Duke O et al 1982). Dendritic cells, which may be of central importance as they are specialised for antigen presentation (Steinman RM et al 1981), are reported to be scantily present in the deep tissues associated with clusters of lymphocytes (Poulter LW et al 1982). Small numbers of giant cells (possibly formed by coalescence of macrophages), mast cells and granulocytes are also present (Henderson B et al 1987).

The presence of large numbers of lymphocytes indicates an immune process and lymphocyte populations in rheumatoid synovium have been intensively investigated. Mabs to lymphocyte surface antigens have allowed minute dissection of the sets and subsets of cells present but such work has so far failed to shed light on the driving force behind the propagation of inflammation, much less its
instigation, and has failed even to identify features specific to rheumatoid arthritis. It is accepted that the predominant lymphocyte sub-type present is the T cell with CD4+ (helper) cells outnumbering CD8+ (cytotoxic-suppressor) cells. The CD4:CD8 ratio, however, varies in different areas of the tissue and there is no consensus, different studies reporting very different ratios for similar areas (Kurosaka et al 1983; Duke O et al 1982; Meijer et al 1982). Although T helper cells are present in large numbers in rheumatoid synovial tissue and fluid, whether they play a central role in pathogenesis is not clear. In synovial fluid from rheumatoid joints, for instance, the levels of the T cell products gamma-interferon and IL-2 are low (Firestein GS et al 1987 and 1988). Also, T cells from rheumatoid synovial fluid show a low level of expression of the Tac (interleukin-2 receptor) antigen (Hemler ME et al 1986). These findings may be due to suppression of T cell activity but it may also be the case that T cells in synovial fluid have reached the end of their useful life and those still present in the synovium should be studied instead.

1.2.4.2. Identification of macrophages in synovial tissue

Large numbers of macrophages are present in rheumatoid synovial tissue and they form a heterogeneous population (Hogg N et al 1985). A variety of methods is available for identifying macrophages in tissue; unfortunately none is wholly satisfactory. The traditional marker for cells of the monocyte-macrophage lineage, and still the most widely used, is the presence of non-specific esterase activity (Yam LT et al 1971). This enzyme may, however, be present in certain non-macrophage cells, such as plasma cells and the endothelium of high endothelial venules, and, more importantly, may not as a marker be inclusive of all macrophages. Other macrophage markers, CD14 and the epitope of the Mab RFD7 (which has no CD designation as yet), have also been shown to be inadequate (Broker BM et al 1990). CD68, as recognised by Mab EBM11, is reported to mark all monocytes and macrophages in all tissues so far studied (which did not
include synovium) (Kelly PMA et al 1988). Mab EBM11 appears to bind to no other cells, with the exception of weak binding to proximal tubular cells in the kidney. CD68 clearly represents an advance on previous macrophage markers in terms of both sensitivity and specificity.

1.2.4.3. Identification of dendritic cells in synovial tissue
Dendritic cells are held to be the primary antigen-presenting cells in tissue (Steinman RM et al 1981) and their identification in inflamed tissue is of great interest as their presence is evidence for antigenic drive to the inflammatory process. They are characterised on ultrastructural criteria and identification by light microscopy can be difficult; in particular, they are morphologically similar to macrophages. Mab RFD1 (which recognises a DQ-related epitope not yet assigned a CD number) binds preferentially to dendritic cells (Poulter LW et al 1986). Previous studies used RFD1 in conjunction with the macrophage marker RFD7 and showed no overlap between the two markers, suggesting RFD1 reliably distinguished dendritic cells from macrophages (Poulter LW et al 1986). The advent of CD68 has, however, thrown doubt on the reliability of RFD7 as a pan-macrophage marker (see 1.2.4.2.), necessitating reassessment of RFD1 as a marker of dendritic cells.

1.2.5. Changes in components of the extracellular matrix in rheumatoid arthritis
Cellular features of synovial inflammation have been extensively studied; by comparison, matrix features have been neglected. It is known that secreted products of inflammatory cells act to disrupt the matrix of bone and cartilage, either by direct action or by stimulating the degradative activities of indigenous chondrocytes and bone cells (Fell H et al 1977), but much less is known about effects on synovial matrix.

Fibroblasts synthesise matrix components and there has been much work on the in
vitro synthetic and degradative capabilities of cultured rheumatoid synovial fibroblasts. Hyaluronan synthesis is increased (Castor CW 1971) and an abnormally low molecular weight form is produced (see 1.3.3.4.). Sulphate incorporation is increased (Marsh JM et al 1979), suggesting upregulation of synthesis of sulphated glycosaminoglycans. Collagen is also produced in greater amounts in comparison with normal (Castor CW 1971). The synthetic behaviour of cultured fibroblasts can be modulated by cytokines; the monokines interleukin-1 and tumour necrosis factor-α both stimulate synthesis of hyaluronan and sulphated glycosaminoglycans (Yaron I et al 1989) and of collagenase and prostaglandin E₂ (Dayer J-M et al 1985 and 1986). If inferences can be made from the behaviour of cultured cells to the behaviour of the parent cells in vivo, then these observations suggest that matrix turnover is increased in rheumatoid arthritis and that at least hyaluronan may be produced in abnormal form.

In contrast, there has been little work on the structure of complete rheumatoid synovial extracellular matrix. Fibronectin has been localised in the matrix and cells of the superficial layer of rheumatoid synovium (Mapp PI et al 1985) and in situ hybridisation studies (Waller et al 1992) showed messenger RNA for fibronectin localised to the same region and also to blood vessel walls. Neither study, however, included normal controls for comparison. The distribution of glycosaminoglycans and collagen has been little studied.

1.3. CARBOHYDRATES IN HEALTH AND DISEASE

1.3.1. General considerations

Carbohydrates may exist as simple monosaccharides and disaccharides or may form chains from oligosaccharides of 10 residues or less to large polysaccharide polymers or glycosaminoglycans, like hyaluronan, comprising tens of thousands of residues. Oligosaccharides may contain a mixture of simple sugars and amino sugars. Glycosaminoglycans always consist of equal numbers of a simple sugar and
an amino sugar which alternate in sequence. Other large non-glycosaminoglycan polysaccharide polymers, such as starch and glycogen, which have a repeating monosaccharide constituent, and agarose, which has a repeating disaccharide constituent, do not contain amino sugar residues (Lehninger AL 1982).

Monosaccharides, disaccharides, oligosaccharides and glycosaminoglycans, with the possible exception of hyaluronan, the largest glycosaminoglycan, may be found linked to protein by the reducing sugar at one end of the carbohydrate chain. Hyaluronan exhibits a specialised linkage with protein found only in cartilage (Hascall VC et al 1974). Glycoproteins are proteins to which from one to many monosaccharides or oligosaccharide chains are attached. Proteins bearing glycosaminoglycans form proteoglycans. The proteoglycan protein core appears to be specialised as a carrier of glycosaminoglycans, whose properties dominate those of the whole molecule. Lipids, particularly those of the cell membrane, may also bear oligosaccharide chains.

1.3.2. Oligosaccharides
1.3.2.1. The role of the oligosaccharide moiety of glycosylated molecules
Almost all proteins (and many lipids), with the notable exception of albumen, undergo post-translational glycosylation and the attached chains of carbohydrate residues have features in common. These oligosaccharides may be O-linked to serine or threonine residues via N-acetyl-β-glucosamine or N-linked to asparagine residues via N-acetyl-β-glucosamine (GlcNAc) with mannose attached peripherally (see Appendix II). The chains may be simple or branched, with mannose residues occurring at the branch points, and frequently terminate in sialic acid residues (West CM 1986). It is clear that glycoproteins, through their carbohydrate components, may exhibit enormous diversity independently of their protein structure.
The role played by attached carbohydrate chains in the behaviour of individual proteins is complex. The ubiquity of glycosylation points to function(s) which are of fundamental importance but several studies have shown alteration of the carbohydrate or even its total removal had no effect on the recognised activities of the protein concerned (e.g. Davis CG 1986) or, in the case of a membrane protein, on the behaviour of the cell to which it was attached (Stanley P 1984). In these circumstances, carbohydrate moieties may modulate hitherto unrecognised functions of the proteins to which they are attached or have effects too subtle to be detected by present techniques. A further possibility is that these effects are of little relevance in crude in vitro experiments but of great importance in the complex natural environment of a living cell.

Evidence is accumulating, however, for important roles for specific protein-associated carbohydrates in the areas of recognition, adhesion and transport. Within the cell, proteins are routed by means of carbohydrate “adressins”. For example, lysosomal enzymes are directed to lysosomes by means of a mannose-6-phosphate moiety which associates the protein with a specific receptor (Sly WS et al 1982). Also, the acute phase protein α_1_-acid glycoprotein is directed to the hepatocyte surface for secretion by its attached carbohydrates (Docherty PA et al 1985). A receptor for asialoglycoproteins has been identified on macrophages, notably the Kupffer cells of the liver, and may serve to remove effete serum proteins. Accumulated damage leading to exposure of an increasing number of residues normally covered by sialic acid may indicate the age of a protein; the receptor recognises these residues (Ashwell G et al 1982).

Numerous instances of carbohydrate involvement in cell-cell recognition have been identified. Examples are: mouse sperm-egg recognition which involves interaction between galactosyl transferase on the sperm acrosome and GlcNAc on the zona pellucida of the ovum (Shur BD et al 1982); lymphocyte binding to high
endothelial venules, which is inhibited by pre-treatment of lymphocytes with mannose-6-phosphate and fructose-1-phosphate and by glycosidase treatment of the high endothelial venules (Rosen SD 1989); macrophage recognition of apoptotic lymphocytes which involves lectins inhibited by GlcNAc, N-acetylgalactosamine and galactose (Duvall E et al 1985).

Carbohydrates are also important in cell-matrix adhesion, as shown by the following examples: neurite outgrowth and adhesion is modulated by differential sialylation of neural cellular adhesion molecule (N-CAM) (Rutishauser U et al 1985); metastasis as measured by lung colonisation by a mouse melanoma is increased by desialylation of a cell surface glycoprotein (Nabi IR et al 1987); cell adhesion and spreading on laminin requires surface galactose transferases, indicating a role for oligosaccharide components of laminin (Runyan RB et al 1988).

It can be seen that the functions so far identified of the carbohydrate moiety of glycosylated molecules are generally to do with recognition, transport and adhesion. Systematic modification of oligosaccharide side chains may facilitate differential transport and homing of important proteins without changing the basic structure of the protein or interfering with its primary function. Alteration of these side chains in disease may have important consequences.

1.3.2.2. N-acetylgalactosamine (GlcNAc) in rheumatoid arthritis - an example of the alteration of the carbohydrate moiety of a glycoprotein in inflammatory disease

The glycosylation status of glycoproteins may be altered in inflammatory disease, as illustrated by the abnormal glycosylation pattern of serum immunoglobulin G in rheumatoid arthritis (Parekh RB et al 1985) and tuberculosis (Rademacher TW et al 1988). Immunoglobulin G bears on its Fc portion two oligosaccharide chains and for the majority of molecules these chains normally terminate in an GlcNAc-
galactose-sialic acid sequence. In rheumatoid arthritis, an increased proportion of immunoglobulin G molecules carry oligosaccharide chains from which the final two residues are missing and which thus terminate in GlcNAc; carbohydrate moieties associated with other parts of the immunoglobulin molecule appear to be unaffected.

It is normally rare for GlcNAc to occupy the terminal position in an oligosaccharide chain, so there are grounds for assuming its exposure in disease to be of significance. First, it has been suggested that the abnormal immunoglobulin G with its exposed GlcNAc is prone to aggregation and that these aggregates are in some way immunogenic (Parekh RB et al/1985). Secondly, the abnormally exposed GlcNAc may itself be a target for antibody binding; antibodies to streptococcal cell wall peptidoglycan-polysaccharide polymers, which contain a high percentage of GlcNAc residues, have been reported in rheumatoid arthritis (Johnson PM et al 1984) and may cross-react with oligosaccharides bearing GlcNAc. Thirdly, if a function of the carbohydrate moiety of a glycoprotein is transport and homing (see 1.3.2.1.), then alteration in the structure of this moiety would presumably interfere with this function.

It is unclear how these abnormal glycosylation patterns arise. Reduced galactosyl transferase activity has been reported in B lymphocytes from patients with rheumatoid arthritis (Axford JS et al 1987), suggesting a defect in synthesis. There is also evidence for post-secretory modification by the action of inflammatory mediators such as oxygen-derived free radicals (Griffiths HR et al 1989). If this, presumably random, mechanism operates to any extent, then many tissue and serum glycoproteins could be so modified in the presence of inflammation (although some may be more susceptible than others). If, on the other hand, the modifications are restricted to certain glycoproteins or certain cell types, this would support the operation of non-random mechanisms, such as altered synthesis and specific enzymatic attack. Nevertheless, abnormal patterns
of glycosylation including expression of terminal GlcNAc, however they arise, may play a central role in disease processes.

It should be noted that although exposure of GlcNAc on normal glycoproteins is rare, it may occur at certain special sites. One such site is the nuclear pore of mammalian cells, the proteins of which uniquely carry an O-linked GlcNAc monosaccharide. It is thought that these glycoproteins are involved in the transport of proteins and RNA through the nuclear pore (Hanover JA et al 1987; Starr CM et al 1990).

1.3.3. Glycosaminoglycans

1.3.3.1. General considerations

The polysaccharide polymers, glycosaminoglycans, are constituents of the extracellular matrix of all tissues (Hay ED 1991). They fall into four classes and are formed from long unbranched chains of repeating disaccharide units, each of which comprises a simple sugar and an aminosugar (see Appendix III). Hyaluronan, for example, contains GlcNAc and glucuronic acid in β(1->4) linkage. It is the only glycosaminoglycan to be found free; all others occur in the matrix bound by the reducing end of the molecule to proteins, known as "core proteins", to form proteoglycans which exhibit wide diversity in structure and size. Cartilage proteoglycan, for example, consists of a core protein bearing hundreds of chondroitin sulphate and keratan sulphate chains with a resultant molecular mass for the whole complex of greater than 1 x 10^6 daltons. These large complexes are then linked by a specialised binding region to long chains of hyaluronan to form the even larger cartilage proteoglycan aggregate (Hascall VC et al 1974). Other proteoglycans, such as decorin, may consist only of a small protein moiety bearing a single glycosaminoglycan chain. Besides glycosaminoglycans, core proteins may also bear a variable number of N- and O-linked oligosaccharides.
1.3.3.2 Hyaluronan

Hyaluronan is of special interest in this study as 50% of its constituent monosaccharides are GlcNAc residues. It is the most widely distributed glycosaminoglycan, being present in virtually all tissues, including synovium, and is particularly plentiful at sites of active remodelling, as in the embryo (Toole BP 1991) and healing wounds (Bentley JP 1968). It is responsible for the viscosity of synovial fluid and forms the bulk of vitreous humour in the eye and Wharton's jelly in the umbilical cord.

Hyaluronan molecules are long, unbranched chains in which the sugars glucuronic acid and GlcNAc alternate (see Appendix III); this structure is conserved from streptococci to mammals. The molecules are large (molecular mass up to $6 \times 10^6$ daltons) and in aqueous solution a system of intramolecular hydrogen bonding and bridging by water molecules gives rise to the secondary structure of a two-fold helix (Scott JE 1992). Early work suggested that, when unconstrained in aqueous solutions of up to 0.04%, hyaluronan molecules occupied non-overlapping domains of up to 0.5 microns in diameter, with significant aggregation occurring only at higher concentrations (Hadler NM 1981). Recently, however, the techniques of rotary shadowing-electron microscopy and computer simulation have shown aggregation of high-molecular weight hyaluronan into extensive meshworks at concentrations as low as 0.001% (Scott JE et al 1991). This tendency to aggregation, together with the stiffness of the secondary structure, is responsible for the marked viscosity of hyaluronan solutions.

Hyaluronan and other glycosaminoglycans help to maintain aqueous channels in tissue matrix for the diffusion of water-soluble molecules (Scott JE 1989). Hyaluronan may also modulate aspects of cellular behaviour and the particular effect observed appears to depend upon the molecular weight species of hyaluronan concerned; native, high-molecular weight hyaluronan appears to be generally
inhibitory whereas low-molecular weight fragments have stimulatory effects which may be relevant to the propagation of inflammation (see 1.3.3.4.).

1.3.3.3. Chondroitin sulphates
Chondroitin sulphate, like hyaluronan, is widely distributed. Several differently sulphated forms occur naturally. Chondroitin-4-sulphate, chondroitin-6-sulphate, multiply-sulphated forms and an unsulphated form have all been described. Chondroitin sulphate appears to occur only as proteoglycan and many chondroitin sulphate proteoglycans, of varying size and carrying from one to many glycosaminoglycan chains, have been identified. As a group, chondroitin sulphate proteoglycans are ubiquitous but individual sulphation forms (Sorrell JM et al 1990; Caterson B et al 1990) and individual proteoglycans (Bianco P et al 1990) show restricted distribution.

Chondroitin sulphate molecules comprise alternating residues of glucuronic acid and N-acetylgalactosamine (see Appendix III) but the chains never achieve the length of hyaluronan molecules. The molecules, like all glycosaminoglycans, are negatively charged and attract water but the specific functions of individual molecular species are largely unknown.

1.3.3.4. Alteration of glycosaminoglycans in disease
There is no pathological condition known in which there are inherited defects of hyaluronan metabolism, probably because the central role played by hyaluronan in embryogenesis would render such a condition lethal soon after conception. Indeed, in vitro work on fibroblasts has shown that hyaluronan is essential for cell detachment and separation after mitosis (Brecht M et al 1986). The metabolism of other glycosaminoglycans is abnormal in the mucopolysaccharidoses, a family of inherited conditions each of which is associated with a deficiency of a specific degradative enzyme. Keratan sulphate, dermatan sulphate, heparan sulphate and
occasionally chondroitin sulphate accumulate, either singly or in combination, leading to a series of clinical patterns dominated by profound abnormalities of musculo-skeletal and connective tissue structures (Wilson JD et al 1991).

Acquired diseases associated with increased serum hyaluronan include inflammatory and malignant conditions, in which there is overproduction of hyaluronan, and diseases of the liver and kidney, which interfere with excretion (Engstrom-Laurent A 1989). In certain malignant diseases, both hyaluronan and its principal receptor, CD44 (see 1.3.4.), appear to be intimately involved in local tumour invasion and metastasis (Knudson W et al 1989; Gunthert U et al 1991).

In rheumatoid arthritis, there is evidence for increased production of hyaluronan. Serum hyaluronan is elevated in patients with rheumatoid arthritis (Engstrom-Laurent A et al 1985a). Fibroblasts cultured from rheumatoid synovium show greatly increased synthesis of hyaluronan when compared with fibroblasts from normal synovium (Castor CW 1971).

The hyaluronan produced in rheumatoid arthritis is of abnormally low molecular weight (Vuorio E et al 1982; Dahl IMS et al 1985) and the molecules may be further fragmented by the action of inflammatory mediators such as free radicals (McNeil JD et al 1985). Indeed, hyaluronan may serve a protective role as a scavenger of free radicals, undergoing depolymerisation in the process. There is evidence that such low molecular weight species of hyaluronan may stimulate elements of the inflammatory process, such as angiogenesis (Kumar S et al 1987), and modulate other aspects of cell behaviour such as proliferation (Goldberg RL et al 1987), lymphocyte transformation (Anastassiades T et al 1984) and IL-1 production by macrophages (Hiro D et al 1986). Low molecular weight species of hyaluronan may therefore play a role in perpetuating the inflammatory process.
Changes in other glycosaminoglycans in rheumatoid arthritis have been little studied. Indeed, changes in individual glycosaminoglycans in disease are generally poorly documented, although changes in total glycosaminoglycan/proteoglycan content have been widely investigated (Varma RS et al 1982). Arterial wall glycosaminoglycan content and distribution, for example, is markedly altered in atheromatous disease (Stevens RL et al 1976). Age-related changes have been documented in a variety of tissues including articular cartilage (Roughly PJ et al 1980) and skin (Sobel H et al 1956). In both tissues, the total glycosaminoglycan/proteoglycan content decreases with age and, in cartilage, the size of the proteoglycan monomer also decreases.

Osteoarthritis and progressive systemic sclerosis are the most extensively investigated rheumatic diseases. Osteoarthritic cartilage has a lower content of chondroitin sulphate than normal and the proteoglycan monomers are smaller than those in age-controlled normals (Hardingham TE et al 1990). Serum keratan sulphate has been proposed as a marker for the degree of cartilage damage (Thonar E J-M A et al 1985) but its reliability has been thrown in doubt by a large well-controlled study which found levels in osteoarthritis to be no different from normal and, intriguingly, levels in rheumatoid arthritis to be lower than normal (Spector TD et al 1992). In progressive systemic sclerosis there is increased skin content of glycosaminoglycan and fibroblasts cultured from affected skin show greatly increased synthesis of glycosaminoglycan compared to normal (Whiteside TL et al 1982). Serum levels are also elevated (Engstrom-Laurent A et al 1985b).

Heparan sulphate is the predominant glycosaminoglycan in basement membrane (Schittny JC et al 1989). Alteration in kidney content of heparan sulphate has been shown in lupus nephritis (van den Born J et al 1992) and diabetic nephropathy (Rohrbach DH et al 1982), both diseases in which the glomerular basement membrane becomes disorganised. Arterial injury damages the heparan
sulphate-rich endothelial basement membrane, releasing bound growth factors such as basic fibroblast growth factor (Bashin P et al 1989) and this may be of relevance in the development of atheromatous disease.

1.3.4. CD44 as a cell-surface receptor for hyaluronan and chondroitin sulphate
Studies on cellular interactions with hyaluronan have identified a hyaluronan receptor present on the surface of a wide variety of cell types. The receptor is an 85 kD glycosylated transmembrane protein (Underhill CB et al 1987) which interacts internally with actin filaments of the cytoskeleton (Lacy BE et al 1987). It binds to a hexasaccharide sequence of hyaluronan and also binds, albeit with lower affinity, to chondroitin sulphate. Affinity is greatest for high-molecular weight hyaluronan, suggesting that a single large hyaluronan polymer may bind simultaneously to several receptors (Underhill CB et al 1980). Other cell surface receptors may also exist, as suggested by the fact that several cell types known to bind hyaluronan do not express the 85 kD protein (Underhill CB 1989).

Recent evidence suggests that the 85 kD hyaluronan receptor is identical with CD44 (Aruffo A et al 1990; Culty M et al 1990). CD44 (also known as the Hermes antigen, Pgp-1 and ECMR-III) is an adhesion molecule with a wide range of specificities and found on a wide range of cell types. It is a multi-domain glycoprotein (see Appendix IV), with N- and O-linked oligosaccharides and chondroitin sulphate chains distributed along its length. A peripheral region of the molecule shows some sequence homology with cartilage link protein and the binding region of cartilage core protein (Goldstein LA et al 1989; Stamenkovic I et al 1989), which are structures specialised for binding to hyaluronan. Functional studies demonstrate binding to hyaluronan and to chondroitin sulphate (Aruffo A et al 1990); CD44 on lymphocytes may play a role in binding to high endothelial venules (Jalkanen S et al 1987).
In immunohistochemical studies of a range of normal tissues (which did not include synovium), Mabs to the 85 kD hyaluronan receptor showed binding patterns strikingly similar to those seen with Mabs to CD44 (Flanagan BF et al 1989; Underhill CB 1989). Distributions in diseased tissues are presently unknown.

1.4. LOCALISATION OF CARBOHYDRATES IN TISSUE

1.4.1. General considerations

Histological studies have traditionally concentrated on the presence and distribution of cell types and these studies have been greatly aided by the development of Mabs to cell-specific markers. Localisation by histochemical techniques of non-cellular tissue components may also yield valuable information as tissue matrix exhibits a marked heterogeneity when individual components are identified (eg Bartholomew JS et al 1982; Couchman JR et al 1984; Craig FM et al 1987; Sorrell JM et al 1990). Morphological studies supply a snapshot of tissue structure in time; while they cannot give direct evidence of function the discovery of restricted distributions may nevertheless suggest specific roles and functions for individual matrix components. Further, a particular component may represent a small fraction of the matrix when analysed biochemically but be restricted in its distribution to certain important sites in the tissue. For example, the non-fibrillar Type IV collagen forms a very small percentage of the collagenous content of most tissues but is restricted to basement membranes where it forms a polymeric network, apparently for the attachment and support of other basement membrane components (Schittny JC et al 1989).

Tissue carbohydrates can be identified by traditional histochemical methods such as the periodic-acid-Schiff reaction but such methods, although easily performed, are crude instruments of detection. The use of specific binding proteins (see 1.4.2. and 1.4.3.), such as lectins and antibodies, allows much greater sensitivity and selectivity.
1.4.2. Localisation of oligosaccharides in tissue

A useful method of identifying oligosaccharides in whole tissue sections is by lectin-binding (Leatham A 1986). Lectins are naturally-occurring carbohydrate-binding proteins of non-immune origin found in plants and animals. They bind to oligosaccharides of various chain lengths and composition and generally bind more strongly to complex rather than simple sugars. Few, however, have an appropriately narrow range of specificities to be of use in identifying particular carbohydrates (Weiner LE et al/1986) and they are most often used to demonstrate differences in the expression of (poorly specified or unspecified) carbohydrates in diseased tissue and its normal counterpart and between diseases (e.g. Zschabitz A et al 1991). *Ulex europaeus* lectin Type I is exceptional in being specific for the monosaccharide L-fucose. L-fucose is restricted in most human tissues to glycoproteins carried by endothelial cells, making Ulex a useful marker of blood vessels in tissue sections (Wilkinson LS et al 1989). Another, relatively specific, lectin is *Bandeiraea simplicifolia* GS II which binds to a group of oligosaccharides terminating in GlcNAc.

Lectins are generally slightly less convenient to use in histochemistry than antibodies but, unfortunately, few useful anti-carbohydrate antibodies are available. An exception is Mab GN7, raised against the polysaccharide-peptidoglycan component of streptococcal cell wall, which recognises terminal GlcNAc (Rook GAW et al 1988).

1.4.3. Advances in the methods of localisation of glycosaminoglycans in tissue

Cationic dyes, such as Alcian blue and ruthenium red, precipitate the negatively-charged glycosaminoglycans by ionic bonding. Histochemical localisation of glycosaminoglycans and proteoglycans became possible with the development of the method of differential staining under controlled electrolyte conditions, in which Alcian blue stains with increasing selectivity as the magnesium chloride
concentration in the staining solution is raised (Scott JE et al 1965). Digestion of tissues with specific degradative enzymes further increases the selectivity of the staining (Bartholemew JS et al 1982). These methods are, however, cumbersome and inflexible. They do not allow a detailed assessment of tissue architecture and double labelling, for example, with Mabs or lectins, is not possible. Another disadvantage is that the presence of individual glycosaminoglycans must be inferred from absence of staining after treatment of the tissue with the relevant specific enzyme; tissues containing several different glycosaminoglycans with overlapping distributions may give uninterpretable results. For ultrastructural localisation, colloidal iron binding has been used (Roy S et al 1967) but, again, this binds to all glycosaminoglycans and enzymatic treatment is required to increase specificity.

More recently, Mabs have been raised to epitopes on each of the sulphated glycosaminoglycans and these have proved useful in localisation studies (Couchman JR et al 1984; Craig FM et al 1987; Sorrell JM et al 1990). Attempts to raise antibodies to hyaluronan, however, have proved in the main unsuccessful. As hyaluronan is distributed with unaltered structure throughout life forms from bacteria to mammals and has been shown to be involved in the separation of cells after mitosis (Brecht M et al 1986), its peculiarly non-antigenic nature is not surprising. A Mab raised against placental extract (Sunderland CA et al 1985) is marketed commercially as an anti-hyaluronan reagent but its specificity and sensitivity have not been established.

Lack of specific reagents suitable for histochemistry has hampered assessment of the morphological distribution of hyaluronan in tissue. The recent development of a specific probe, derived from the hyaluronan-binding region of cartilage proteoglycan monomer core protein, for hyaluronan (Ripellino JA et al 1985) represents a major advance. Binding region binds non-covalently with high affinity and specificity to decasaccharide segments of the hyaluronan molecule.
(Hardingham TE et al/1973); for binding to occur the molecule must possess unoccupied sites accessible to the probe. The binding region probe has been used, with apparent success, to localise hyaluronan in normal human skin (Tammi R et al/1988) and various animal tissues (Ripellino JA et al/1985; Underhill CB 1989).

A study of carbohydrates in synovial disease could reasonably begin with their morphological distribution in tissue. GlcNAc and GlcNAc-containing carbohydrates, such as hyaluronan, may play a special role in rheumatoid arthritis and the finding of specific restricted distributions would support this.
2. MATERIALS AND METHODS

2.1. TISSUES AND CELLS

2.1.1. Tissue collection, processing and storage

Adult diseased human tissues were obtained fresh from material removed at surgery performed for clinical indications. Diseased synovium in particular was obtained from patients undergoing arthroscopy, prosthetic arthroplasty and synovectomy. Patients with rheumatoid arthritis all satisfied the revised Arthritis and Rheumatism Association criteria for the classification of the disease (see Appendix I). Patients with osteoarthritis all had a typical pattern of joint involvement with typical radiological changes and no evidence of systemic inflammatory disease.

Normal tissues were obtained from several sources. Normal synovium, skin and muscle were obtained from patients undergoing high-level limb amputation for proximal malignancy. Synovium was extracted from clinically normal joints at least 30 cm distal to the tumour site. Normal skin was also obtained from volunteers undergoing forearm punch biopsy for a separate study for which ethical approval had been obtained and formal consent given. Normal skeletal muscle was also obtained from volunteers undergoing percutaneous needle biopsy of the quadriceps for a separate study for which ethical approval had been obtained and formal consent given. Specimens of normal placenta and umbilical cord were obtained from discarded material.

All tissues were processed within 2 hours of removal. Tissues were dissected free of surrounding fat and capsule and cut into blocks approximately 5 mm square. Synovial samples for enzymatic disaggregation (see 2.1.2.) were processed immediately. Specimens for histochemistry were snap-frozen in a bath of isopentane and solid carbon dioxide and stored in air-tight vials at -70 deg C.
Tissue sections were cut at a thickness of 5 - 7 microns on a cryostat (SLEE, UK) and orientated onto dry glass microscope slides previously coated with 0.5% poly-L-lysine (Sigma Chemical Company Ltd, UK) to promote adherence. Slides were dried under a cool fan, wrapped in tinfoil and stored in air-tight containers at -70 deg C.

2.1.2. Enzymatic disaggregation of rheumatoid synovium

Synovium was minced finely with scissors and digested for 2 hours at 37 deg C with a mixture of 30 mg of collagenase (Worthington Biochemical Corporation, USA) and 3 mg of DNAase (Sigma Chemical Company Ltd, UK) in 20 ml of calcium- and magnesium-free Hank's balanced salt solution (Gibco, UK). The disaggregated tissue was sieved free of debris, using sieves of progressively finer mesh, and the resultant cell suspension prepared for cytopinning (see 2.1.4.).

2.1.3. Separation of monocytes from peripheral blood

Special reagents:

a) Phosphate-buffered saline (PBS)

2 g KCl, 2 g \(KH_2PO_4\), 11.48 g \(Na_2HPO_4\), 80 g NaCl were dissolved in distilled water, made up to 10 litres and the pH adjusted to 7.4.

b) Culture medium

RPMI (Gibco, UK) with 10% foetal calf serum, 25 mM Hepes (Gibco, UK), 2mM glutamine, 100 U/ml penicillin and 0.1mg/ml streptomycin

40 ml of peripheral blood from a normal donor was collected into a sterile tube containing 1 ml of perservative-free heparin (CP Pharmaceuticals, UK). The blood was layered in 10 ml aliquots onto 10 ml of Lympho-paque (Nygaard, Norway) in separate tubes and centrifuged at 2200 rpm for 20 minutes. The mononuclear cell layers were removed with a sterile Pasteur pipette, pooled and the cells washed twice in RPMI. The resultant pellet was suspended in 1 ml RPMI.
and the cells counted on a haemocytometer. The suspension was made up to 10 ml with culture medium. 5 ml was pipetted on each of two plastic petri dishes and incubated at 37 deg C for 2 hours. Non-adherent cells were removed by pipette and discarded. Adherent cells were rinsed twice with RPMI then detached by gentle scraping with a rubber policeman. Adherent cells were pelleted and resuspended in PBS for cytopinning (see 2.1.4.).

2.1.4. Preparation of cytopins

Cell suspensions were adjusted to $1 \times 10^5$ cells/ml with PBS and 100 microlitre aliquots centrifuged at 500 rpm for 10 minutes onto microscope slides using a Shandon Cytospin 2. The cytopins were air-dried and stored wrapped in foil at -70 deg C.

2.2. PRIMARY REAGENTS

2.2.1. Antibodies

Table 2.1.

RABBIT POLYCLONAL ANTIBODIES USED IN THIS STUDY

<table>
<thead>
<tr>
<th>DESIGNATION</th>
<th>SOURCE</th>
<th>WORKING DILUTION</th>
<th>SPECIFICITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-fibrin</td>
<td>Dako A080</td>
<td>1:2000</td>
<td>fibrin, fibrinogen</td>
</tr>
<tr>
<td>anti-human IgG</td>
<td>Dako A090</td>
<td>1:200</td>
<td>human IgG</td>
</tr>
<tr>
<td>anti-fibronectin</td>
<td>Dako A245</td>
<td>1:2000</td>
<td>fibronectin</td>
</tr>
</tbody>
</table>

Key: IgG = immunoglobulin G
**Table 2.2.**

**MONOCLONAL ANTIBODIES USED IN THIS STUDY**

<table>
<thead>
<tr>
<th>DESIGNATION</th>
<th>SOURCE</th>
<th>WORKING DILUTION</th>
<th>Ig CLASS</th>
<th>SPECIFICITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN7</td>
<td>G Rook</td>
<td>1:200</td>
<td>IgM</td>
<td>Terminal N-acetylglucosamine</td>
</tr>
<tr>
<td>EBM11</td>
<td>Dako M718</td>
<td>1:200</td>
<td>IgG</td>
<td>CD88 (monocytes/macrophages)</td>
</tr>
<tr>
<td>RFD1</td>
<td>L Poulter</td>
<td>1:4</td>
<td>IgM</td>
<td>DQ-related (dendritic cells)</td>
</tr>
<tr>
<td>RFD7</td>
<td>L Poulter</td>
<td>1:10</td>
<td>IgG</td>
<td>Macrophages</td>
</tr>
<tr>
<td>anti-CD3</td>
<td>Dako M756</td>
<td>1:10</td>
<td>IgG</td>
<td>CD3 (pan-T lymphocytes)</td>
</tr>
<tr>
<td>anti-CD22</td>
<td>Dako M738</td>
<td>1:10</td>
<td>IgG</td>
<td>CD22 (pan-B lymphocytes)</td>
</tr>
<tr>
<td>2B6</td>
<td>B Caterson</td>
<td>1:100</td>
<td>IgG</td>
<td>Chondroitinase ABC-digested Ch4S/DS PG and chondroitinase AC-digested DS PG*</td>
</tr>
<tr>
<td>3B3</td>
<td>B Caterson</td>
<td>1:100</td>
<td>IgM</td>
<td>Chondroitinase ABC-digested Ch6S PG*</td>
</tr>
<tr>
<td>F.10.44.2</td>
<td>J Fabre</td>
<td>1:2500</td>
<td>IgG</td>
<td>CD44</td>
</tr>
<tr>
<td>NDOG1</td>
<td>Serotec MCA 277</td>
<td>1:10</td>
<td>IgM</td>
<td>hyaluronan**</td>
</tr>
</tbody>
</table>

Key: IgG = immunoglobulin G; IgM = immunoglobulin M; Ch4S = chondroitin-4-sulphate; Ch6S = chondroitin-6-sulphate; DS = dermatan sulphate; PG = proteoglycan

* see 2.4.21.

** NDOG1 was raised against human placental extract (Sunderland CA *et al* 1985) and is marketed commercially as an anti-hyaluronan reagent. Its sensitivity and specificity as a marker for hyaluronan are investigated in this study (see 4.2.4.).
2.2.2. Lectins

*Ulex europaeus* lectin Type 1 (Dako Ltd, UK) was used at a dilution of 1:400.

*Bandeiraea simplicifolia* GS II (Sigma Chemical Company Ltd, UK) was biotinylated (see 2.5.2.) and used within 48 hours at a dilution of 1:10.

2.2.3. Preparation of the binding region probe for hyaluronan

Biotinylated binding region probe was a gift from Dr M T Bayliss, Kennedy Institute for Rheumatology. The method of preparation was, briefly, as follows. Binding region of pig laryngeal cartilage proteoglycan monomer was prepared using a modification (Bonnet F et al 1985) of the method of Ripellino (Ripellino JA et al 1985). Proteoglycan aggregates were purified from a 4-molar guanidine hydrochloride extract of the tissue by equilibrium caesium chloride density gradient centrifugation. After sequential digestion of the aggregate with chondroitinase ABC (ICN Biochemicals, USA) and trypsin (Sigma Chemical Company Ltd, UK), binding region was isolated by gel exclusion chromatography on Sepharose CL-6B (Pharmacia LKB, Biotechnology, Sweden) followed by size exclusion high performance liquid chromatography on a TSKW 4000 column (Anachem, UK). Before conjugation with biotin-N-hydroxysuccinamine ester (Sigma Chemical Company Ltd, UK), binding region was reassociated with exogenous hyaluronan (human umbilical cord hyaluronan from Sigma Chemical Company, UK) to protect its functional binding site. It was then separated from hyaluronan on a TSKW 4000 column, eluted with 4-molar guanidine hydrochloride. The lyophilised protein was stored at -20 deg C as a 1 mg/ml solution in PBS. The working dilution was 100 microgram/ml in PBS.

2.3. SECONDARY REAGENTS

2.3.1. Bridging antibodies

Swine anti-rabbit immunoglobulins (Dako Z196) were used at a dilution of 1:200.
### 2.3.2. Conjugated antibodies

**Table 2.3.**

**CONJUGATED ANTIBODIES USED IN THIS STUDY**

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>SOURCE</th>
<th>WORKING DILUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>R anti-M horseradish peroxidase</td>
<td>Dako P260</td>
<td>1:30</td>
</tr>
<tr>
<td>alkaline phosphatase-anti-alkaline phosphatase complexes (raised in mouse)</td>
<td>Dako D651</td>
<td>1:50</td>
</tr>
<tr>
<td>G anti-M IgG fluorescein isothiocyanate</td>
<td>Sera-Lab SBA 1030-02</td>
<td>1:20</td>
</tr>
<tr>
<td>G anti-M IgM tetramethyl rhodamine isothiocyanate</td>
<td>Sera-Lab SBA 1020-03</td>
<td>1:20</td>
</tr>
<tr>
<td>R anti-(<em>Ulex europaeus</em>) lectin Type 1 horseradish peroxidase</td>
<td>Dako P289</td>
<td>1:100</td>
</tr>
<tr>
<td>G anti-M IgG biotin</td>
<td>Amersham RPN 1177</td>
<td>1:50</td>
</tr>
<tr>
<td>G anti-M IgM biotin</td>
<td>Amersham RPN 1176</td>
<td>1:50</td>
</tr>
</tbody>
</table>

Key: Igs = immunoglobulins; IgG = immunoglobulin G; IgM = immunoglobulin M; R = rabbit; M = mouse; G = goat
2.3.3. Streptavidin conjugates

Table 4

STREPTAVIDIN CONJUGATES USED IN THIS STUDY

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>SOURCE</th>
<th>WORKING DILUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>horseradish peroxidase</td>
<td>Dako P397</td>
<td>1:400</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td>Dako D396</td>
<td>1:200</td>
</tr>
<tr>
<td>tetramethyl rhodamine isothiocyanate</td>
<td>Sera-Lab 9BA 7100-03</td>
<td>1:20</td>
</tr>
</tbody>
</table>

2.4. HISTOCHEMICAL PROCEDURES

2.4.1. General considerations

All histochemistry was performed on cryostat sections of tissue. Slides retrieved from storage at -70 deg C were brought to room temperature before unwrapping in order to minimise damage to sections by condensation. Unless otherwise stated, the following held for all histochemical procedures. Sections were fixed by immersion for 10 minutes in acetone kept at -20 deg C. After fixation, air-drying and rehydration in PBS, all sections were treated for 10 minutes with 10% normal serum of the same species as the secondary antibody to block non-specific binding. Sections were blotted free of surplus serum before incubation with the primary antibody. Washes were for 5 minutes in PBS, reagents were diluted in PBS (which for secondary reagents contained 1% normal human serum) and all incubations were with 25 microlitre aliquots for 30 minutes in a humidified chamber at room temperature. Reagent controls were included with each experiment.
2.4.2. Peroxidase developed with diaminobenzidine

*Special reagents:*

Diaminobenzidine developing reagent

100 mg of diaminobenzidine tetrahydrochloride (Sigma Chemical Company Ltd, UK) was dissolved in 50 ml PBS and stored as stock in aliquots of 0.5 ml at -20 deg C.

Fixed, rehydrated, blocked sections were incubated with the relevant antibody. Sections were washed three times and then treated for 5 minutes with 0.5% hydrogen peroxide in methanol to block endogenous peroxidase activity. After three washes, sections were incubated with an appropriate horseradish peroxidase-conjugated antibody. Sections were washed three times and incubated for 2-10 minutes with 0.5 ml diaminobenzidine stock solution added to 1.5 ml PBS and 10 microlitres 3% hydrogen peroxide. The reaction is sensitive so fresh batches of substrate were first tested in a control system (eg using monoclonal antibody (Mab) EBM11, which binds to the monocyte/macrophage marker CD68, on tonsil) to assess the appropriate time for optimal colour development. The reaction gives rise to a yellow-brown product insoluble in water and alcohol. The reaction was halted by immersion of slides in PBS. Sections developed with diaminobenzidine were then dehydrated in graded ethanols (70%, 90%, 100%), cleared in CNP 30 (trichloroethane/trichlorethylene from Penetone, UK) and mounted in DePeX (British Drug Houses, UK).

2.4.3. Peroxidase developed with ethylaminocarbazole

*Special reagents:*

a) Acetate buffer

0.2M sodium acetate brought to pH 5.0 with acetic acid

b) Ethylaminocarbazole developing reagent

10 mg 3-amino-9-ethylcarbazole (Sigma Chemical Company Ltd, UK) was
dissolved in 5 ml dimethylsulphoxide (Sigma Chemical Company Ltd, UK) in a
glass container and stored as stock wrapped in foil at 4 deg C.

Sections were processed as above (see 2.4.2.) but incubated with 100 microlitres
of ethylaminocarbazole stock solution added to 900 microlitres of acetate buffer
containing 10 microlitres 3% hydrogen peroxide for 10 minutes in place of
diaminobenzidine. Sections developed with aminoethylcarbazole, which yields a
reddish-brown reaction product, were mounted in Apathy's aqueous medium
(British Drug Houses, UK) without dehydration as the reaction product is alcohol-
soluble.

2.4.4. Alkaline phosphatase-anti-alkaline phosphatase

Special reagents:

a) Tris-buffered saline (TBS)
   0.05 M Tris (Tris[hydroxymethyl]amino-methane) and 0.05 M Tris
   hydrochloride (both from Sigma Chemical Company Ltd, UK), pH 8.2

b) Developing reagent
   10mg naphthol AS-MX phosphate disodium salt (Sigma Chemical Company Ltd,
   UK) was dissolved in 1 ml dimethylformamide (British Drug Houses, UK)
   in a glass container. 20ml TBS was added and the solution stored as stock at
   4 deg C.

After initial processing and incubation with the primary antibody, sections were
washed three times then incubated with either rabbit anti-mouse immunoglobulins
followed by alkaline phosphatase-anti-alkaline phosphatase complexes raised in
mouse for mouse primary antibodies or swine anti-rabbit immunoglobulins
alkaline phosphatase conjugate for rabbit primary antibodies. Sections were
washed three times in TBS and incubated for 5 minutes with 1 ml developing
reagent stock solution to which was added 5 mg fast red or fast blue (both from
Sigma Chemical Company Ltd, UK) and 10 microlitres 0.1M levamisole hydrochloride (Sigma Chemical Company Ltd, UK) (alkaline phosphatase-anti-alkaline phosphatase complexes contain enzyme of intestinal origin; levamisole blocks all endogenous non-intestinal enzyme). The reaction was terminated by immersion of slides in TBS. The reaction product of fast red is magenta in colour and that of fast blue, indigo. The reaction product is soluble in alcohol so sections were mounted in Apathy's aqueous medium without dehydration.

2.4.5. Double staining with monoclonal antibodies using peroxidase and alkaline phosphatase-anti-alkaline phosphatase

The method for peroxidase (see 2.4.2.) was followed up to and including colour development, except that serum from an irrelevant species was chosen for blocking of non-specific binding sites. The method for alkaline phosphatase-anti-alkaline phosphatase (see 2.4.4.) was then followed. Development with fast red gives the better contrast with diaminobenzidine. Sections were mounted in Apathy's aqueous medium.

2.4.6. Non-specific esterase activity

Special reagents:

a) Non-specific esterase buffer

\[ 67.5 \text{ ml } 0.066 \text{ M } \text{KH}_2\text{PO}_4 \text{ was added to 432 ml } 0.066 \text{ M } \text{Na}_2\text{HPO}_4, \text{ pH 7.6} \]

b) Pararosaniline

2 g pararosaniline hydrochloride (Sigma Chemical Company Ltd, UK) was dissolved in 50 ml 2N hydrochloric acid.

Frozen sections of tissue were fixed in cold acetone and rehydrated in PBS. 10 mg alpha-naphthylacetate (Sigma Chemical Company Ltd, UK) was dissolved in 0.5ml acetone in a glass vial. 0.3 ml of pararosaniline and 0.3 ml of a freshly-made 4% solution of sodium nitrite in distilled water were mixed for 1 minute in a glass
vial. The alpha-naphthylacetate solution was added to 9 ml of non-specific esterase buffer and the pararosaniline/sodium nitrite mixture added. The resultant solution was filtered and sections incubated with 25 microlitre aliquots for 8 -10 minutes. Care was taken to prevent overdevelopment of the reaction as this leads to a generalised yellow background. The reaction was terminated by immersion of slides in PBS. Sections were dehydrated in graded alcohols, cleared in CNP 30 and mounted in DePeX.

2.4.7. Double staining for non-specific esterase activity and a monoclonal antibody using alkaline phosphatase-anti-alkaline phosphatase
Non-specific esterase activity was ascertained (see 2.4.6.) first followed by Mab binding visualised by alkaline phosphatase-anti-alkaline phosphatase developed with fast blue (2.4.4.). The two reactions gave rise respectively to brick red and deep blue colour products which were easily distinguished. Sections were mounted in Apathy’s aqueous medium without dehydration.

2.4.8. Ulex europaeus lectin Type 1 staining using immunoperoxidase
Special reagents:

Tris-buffered saline (TBS) with heavy metal ions
60.6 g Tris and 87 g NaCl 10 litres of distilled water with 1 mM magnesium and manganese and calcium chlorides, final pH 7.4. The presence of heavy metal ions enhances binding of the lectin to its carbohydrate ligand.

Fixed sections were rehydrated in TBS with heavy metal ions and incubated for 60 minutes with Ulex made up in the same buffer. Sections were washed three times, blocked with 0.5% hydrogen peroxide in methanol, washed three times and incubated with rabbit anti-Ulex horseradish peroxidase conjugate for 30 minutes, all steps using TBS with heavy metal ions. Sections were then washed three times in PBS and incubated with diaminobenzidine (see 2.4.2.), dehydrated, cleared and
mounted in DePeX.

2.4.9. Double staining with Ulex using immunoperoxidase and a monoclonal antibody using alkaline phosphatase-anti-alkaline phosphatase

Sections were processed as in 2.4.8. up to and including colour development with diaminobenzidine. Sections were then transferred to TBS and processed as in 2.4.4.

2.4.10. *Bandeiraea simplicifolia* GS II (biotinylated) staining using peroxidase

Fixed sections were rehydrated in TBS with heavy metal ions (see 2.4.8.) and incubated with the biotinylated lectin for 60 minutes. Sections were washed three times, blocked with 0.5% hydrogen peroxide in methanol, washed three times, and then incubated with streptavidin horseradish peroxidase conjugate, all steps using TBS with heavy metal ions. Sections were then washed three times in PBS, incubated with diaminobenzidine (see 2.4.2.), dehydrated, cleared and mounted in DePeX.

2.4.11. Immunofluorescence visualisation of monoclonal antibody binding

*Special reagents:*

a) Glycine buffer

14 g glycine and 17 g sodium chloride were dissolved in 800 ml distilled water, the pH brought to 8.6 with 10% sodium hydroxide and the solution made up to 1 litre.

b) DABCO mountant

2.5 g DABCO (1,4-diazabicyclo-[2.2.2.]octane from Sigma Chemical Company Ltd, UK) was added to 30 ml glycine buffer and the pH brought to 8.6 with concentrated hydrochloric acid. 30 ml was added to 70 ml glycerol and the solution stored wrapped in foil at 4 deg C.
Sections were incubated with the Mab and then washed three times in PBS. Goat anti-mouse fluorescein conjugate raised against the mouse immunoglobulin of the appropriate class was prepared at the working dilution, centrifuged at $13 \times 10^3$ rpm for 5 minutes to remove fluorescent precipitated material and applied to the sections. Sections were washed three times in PBS and mounted in the non-fade mountant DABCO which allows storage of slides for up to 3 weeks at 4 deg C without deterioration.

2.4.12. Double immunofluorescence with monoclonal antibodies of differing class
For double immunofluorescence with Mabs of differing class (IgG and IgM), sections were processed as in 2.4.11. except that sections were incubated with the two Mabs of interest simultaneously, followed by simultaneous incubation with goat anti-mouse IgG fluorescein conjugate and goat anti-mouse IgM rhodamine conjugate.

2.4.13. Double immunofluorescence with monoclonal antibodies of same class
For double immunofluorescence with Mabs of the same class, one Mab was used in biotinylated form. Sections were incubated with the first, non-biotinylated, Mab followed by the appropriate goat anti-mouse immunoglobulin fluorescein conjugate (see 2.4.11.). Sections were then incubated for 20 minutes with 20% mouse serum in PBS to block unoccupied binding sites on the secondary antibody. This was followed without washing by incubation with the second, biotinylated Mab followed by streptavidin rhodamine conjugate.

2.4.14. Binding region probe staining method using peroxidase
A range of fixatives was assessed (see 4.2.2.1. and 4.2.2.3.) for use with the binding region probe. Fixed sections were incubated with the binding region probe at a concentration of 100 micrograms/ml in PBS. A time course was performed (see 4.2.2.2.) to ascertain optimum incubation time. After washing three times,
blocking for 5 minutes with 0.5% hydrogen peroxide in methanol and again washing three times, sections were incubated with streptavidin horseradish peroxidase conjugate. After a further three washes, colour was developed using either diaminobenzidine (see 2.4.2.) or ethylaminocarbazole (see 2.4.3.).

2.4.15. Binding region probe staining method using alkaline phosphatase
Sections were processed and incubated with the binding region probe (see 2.4.14.), then washed three times and incubated with streptavidin alkaline phosphatase conjugate. After three washes in TBS, colour was developed using fast blue (see 2.4.4.). Sections were mounted in Apathy's aqueous medium without dehydration.

2.4.16. Binding region probe staining method using immunofluorescence
Sections were incubated with the binding region probe (see 2.4.15.), washed three times, incubated with centrifuged (2.4.11.) streptavidin rhodamine conjugate, washed three times again and mounted in DABCO.

2.4.17. Hyaluronidase treatment of sections
Special reagents:

a) Acetate buffer

0.1M sodium acetate brought to pH 5 with acetic acid

b) Hyaluronidase

100 turbidity-reducing units of Streptomyces hyaluronidase (Calbiochem Corporation, USA) was dissolved in 0.5 ml 0.1 molar acetate buffer in the presence of the protease inhibitors aminocaproic acid (100mM), ethylenediamine tetraacetate (10mM) and benzamidine hydrochloride (5mM) (all from Sigma Chemical Company, Ltd, UK)

Sections were incubated for 4 hours at room temperature with hyaluronidase at 5
turbidity-reducing units/section. Sections were washed in PBS before incubating with the binding region probe. Hyaluronidase from *Streptomyces hyaluronicus*, unlike hyaluronidase from other sources, is specific for hyaluronan and has no action on proteins or other glycosaminoglycans (Ohya T et al 1970). It degrades hyaluronan ultimately to its constituent disaccharides, most of which are probably then lost in washing steps. Its specificity has been assessed for use in histochemistry (Derby MA et al 1978).

2.4.18. Preparation of hyaluronan oligosaccharides
10 mg human umbilical cord hyaluronan (Sigma Chemical Company Ltd, UK) was dissolved in 5 ml PBS and incubated with bovine testicular hyaluronidase (Sigma Chemical Company Ltd, UK) at 4 turbidity-reducing units/mg of hyaluronan for 4 hours at 37 deg C. The enzyme was precipitated by heating at 100 deg C for 5 minutes and the digest was dialysed against distilled water, lyophilised, redissolved in PBS at 2 mg/ml and stored at -20 deg C. This method generates a mixture of oligosaccharides of hyaluronan of varying molecular weight.

2.4.19. Binding region probe specificity controls
Binding region probe at 100 microgram/ml in PBS was incubated with hyaluronan oligosaccharides (see 2.4.18.) or chondroitin sulphate (Sigma Chemical Company Ltd, UK), each at a final concentration of 100 microgram/ml, for 2 hours at room temperature. Sections were incubated with the mixtures and further processed as shown above (see 2.4.14.).

2.4.20. Double immunofluorescence with the binding region probe and a monoclonal antibody
Double immunofluorescence with the binding region probe and a Mab was performed by incubating sections with binding region (see 2.4.14.), followed by the Mab, followed by streptavidin rhodamine conjugate, followed by goat anti-
mouse IgG fluorescein conjugate. Both secondary reagents were centrifuged before use (see 2.4.11.). All steps were separated by three washes and sections were mounted in DABCO.

2.4.21. Chondroitinase ABC and chondroitinase AC digestion of tissues for staining with monoclonal antibodies 2B6 and 3B3

*Special reagents:*

- Tris/acetate buffer
  
  0.1M Tris, 0.1M sodium acetate brought to pH 7.3 with acetic acid

Sections were incubated with chondroitinase ABC or AC (both from ICN Biochemicals, USA) at 0.2 units/ml in Tris/acetate buffer for one hour at 37 deg C and then washed three times in PBS prior to incubating with the anti-chondroitin/dermatan sulphate Mabs 2B6 and 3B3. Both enzymes cleave the glycosaminoglycan chain, leaving a disaccharide stub attached via the specialised link region to core protein, which forms the Mab epitope. Chondroitinase ABC exposes the Mab 2B6 epitopes on chondroitin-4-sulphate and dermatan sulphate and the Mab 3B3 epitope on chondroitin-6-sulphate; chondroitinase AC exposes only the Mab 2B6 epitope on dermatan sulphate.

2.5. MISCELLANEOUS

2.5.1. Dot-blotting

*Special reagents:*

a) Tris buffered saline (TBS) with Tween

  4 g Tris hydrochloride, 9 g sodium chloride, 0.5 ml Tween 20

  (polyoxethylene-sorbitan monolaurate from Sigma Chemical Company, Ltd) in 1 litre, pH 7.6

b) Blocking reagent

  5% bovine serum albumen (Sigma Chemical Company Ltd, UK), 2% gelatin
(Difco Laboratories, USA) and 10% normal goat serum in TBS with Tween

All washes were for 30 minutes in TBS with Tween and all reagents were made up in TBS with Tween. Human umbilical cord hyaluronan and oligosaccharides of hyaluronan (see 2.4.18) were each applied to strips of nitrocellulose paper of mesh size 0.45 micron (Sartorius AG, Germany) in 1 microlitre drops at 1 mg/ml and allowed to dry thoroughly. The strips were incubated with blocking reagent overnight at room temperature on a roller, washed 3 times and then incubated overnight with binding region probe for hyaluronan at 1 microgram/ml and Mab GN7 at 1:2000. The strips were again washed 3 times. The strip exposed to binding region probe was then incubated overnight with streptavidin horseradish peroxidase conjugate at 1:4000 and the strip exposed to Mab GN7 with rabbit anti-mouse horseradish peroxidase conjugate at 1:300. After 3 washes, all strips were incubated with the developing reagent diamnobenzidine (see 2.4.2.) for 30 minutes, washed thoroughly and allowed to dry.

2.5.2. Biotinylation of reagents

The protein to be biotinylated was dissolved at 1 mg/ml in 0.1 M sodium bicarbonate. Biotin-N-hydroxysuccinimide ester (Sigma Chemical Company Ltd, UK) at 1 mg/ml in dimethylformamide (Sigma Chemical Company Ltd, UK) in a glass container was added to the protein solution in a ratio of 1:10 and the reaction allowed to proceed for 30 minutes at room temperature. The solution was dialysed overnight against PBS to remove unbound biotin and low molecular weight by-products. The solution was titrated for use in histochemistry, stored at 4 deg C and used within 48 hours.

2.6. MICROSCOPY

2.6.1. Light microscopy

Light microscopy was performed using a Nikon Optiphot research microscope.
Eyepiece magnification was x10 and objectives were available giving the following magnifications: x4, x10, x20, x40, x60, x100 (oil immersion).

2.6.2. Fluorescence microscopy

Fluorescence microscopy was performed using the above Nikon research microscope with a 100 watt mercury lamp. The excitation method for fluorescein was centred on 495 nanometres with an excitation filter operating at 460-485 nanometres. The excitation method for rhodamine was centred on 546 nanometres with an excitation filter operating at 535-550 nanometres.

2.6.3. Photomicrography

Light photomicrography was performed using a camera attachment to the Nikon research microscope and an Olympus OM-100 automatic camera containing ASA 160 film for colour and ASA 50 film for monochrome. The light source was held at maximum intensity and the exposure time adjusted to 0.05 seconds by means of up to 4 neutral density filters placed in the light path. Fluorescence photomicrography was performed using the above system but with ASA 400 film.

Developing and printing were performed professionally by Colour Processing Laboratories Ltd, London, UK.
3. RESULTS I: TISSUE EXPRESSION OF TERMINAL N-ACETYLGALACOSAMINE (GlcNAc) AND ITS ALTERATION IN INFLAMMATORY DISEASE

3.1. GENERAL INTRODUCTION

Using a variety of histochemical techniques and a specific monoclonal antibody (Mab), GN7, the expression of terminal GlcNAc in synovium was investigated. Normal synovium was compared with rheumatoid tissue and the patterns of cellular (cytoplasmic/membrane and nuclear) and extracellular distribution delineated. Specificity of the changes observed for rheumatoid disease was investigated using synovium affected by non-rheumatoid disease and a variety of non-synovial tissues affected by different inflammatory diseases.

Cytoplasmic/membrane expression of terminal GlcNAc in rheumatoid synovium was investigated in a series of double-labelling experiments to determine whether it was restricted to a particular cell type. Extracellular terminal GlcNAc in rheumatoid synovium was investigated in relation to structural features of the tissue and in relation to the distribution of the selected common glycosylated molecules, fibrin, fibronectin, immunoglobulin G and hyaluronan. Further studies were performed on the relation between terminal GlcNAc, as recognised by Mab GN7, and hyaluronan.

3.2. GENERAL CONSIDERATIONS OF TERMINAL GlcNAc EXPRESSION IN SYNOVIUM

3.2.1. Introduction

Terminal GlcNAc expression in synovium was assessed histochemically using Mab GN7. The specificity of this Mab was checked against the lectin Bandeiraea simplicifolia GS II, which binds to GlcNAc only when it terminates an oligosaccharide chain. Multiple normal and rheumatoid synovial samples were assessed for the presence of cellular and extracellular GlcNAc. The presence of
terminal GlcNAc on synovial cell nuclei was further investigated.

3.2.2. Terminal GlcNAc expression in normal synovium

Samples of synovium (n=10) obtained from normal joints were assessed for terminal GlcNAc expression using Mab GN7. The samples taken were from the knee (n=6), ankle (n=3) and wrist (n=1) and no two samples were from the same joint.

The samples chosen showed varying morphology with intimal layers of one to six cells thickness and subintimal layers of fibrous, adipose and areolar type. Morphology showed no association with joint of origin. In all samples the extracellular matrix was unstained and there was no cytoplasmic or membrane cellular staining (Figure 3.1.). All cells in all samples, however, showed nuclear staining, the intensity of which showed regional variation (see 3.2.4.).

Figure 3.1. Terminal GlcNAc expression in normal synovium. Staining is confined to the nuclei of lining cells (arrow). (Peroxidase developed with diaminobenzidine with haematoxylin counterstain x 20)
3.2.3. Terminal GlcNAc expression in rheumatoid synovium

Samples of rheumatoid synovium (n=10) were assessed for terminal GlcNAc expression using Mab GN7. Samples were obtained from ten separate patients and a variety of anatomical sites (Table 3.1.).

All samples showed staining of cell nuclei (see 3.2.4.) and homogeneous cytoplasmic/membrane staining of a proportion of cells (Figure 3.2.). It was not possible to distinguish membrane staining separately. Cells with homogeneous cytoplasmic/membrane staining were located particularly in the surface layers, around lymphoid aggregates and scattered both singly and in small groups throughout the tissue. The cells were generally large, rounded mononuclear cells with a high cytoplasm-nuclear ratio. A proportion showed multiple cytoplasmic processes. Two samples showed collections of cells with densely stained, granular cytoplasm which appeared to be haemosiderin-laden macrophages. Giant cells also showed dense cytoplasmic/membrane staining for GlcNAc. Extracellular staining, present in a total of eight samples, was of two types: small areas of fine, fibrillar staining scattered sparsely throughout the deep layers and areas of densely stained, coarse reticular pattern of variable size which were associated mainly with surface layers and regions of tissue splitting (see 3.4. and Figure 3.11.). The interstices of the GlcNAc network were frequently occupied by large mononuclear cells. There was no association between the two patterns of extracellular staining, or of either with cellular staining.
## Table 3.1.

**PATTERNS OF TERMINAL GlcNAc EXPRESSION IN RHEUMATOID SYNOVIA**

<table>
<thead>
<tr>
<th>SAMPLE</th>
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<td>+</td>
<td>- +</td>
</tr>
<tr>
<td>87</td>
<td>F 52</td>
<td>wrist</td>
<td>52</td>
<td>+</td>
<td>- - +</td>
</tr>
<tr>
<td>91</td>
<td>F 63</td>
<td>hip</td>
<td>91</td>
<td>+</td>
<td>- + -</td>
</tr>
<tr>
<td>134</td>
<td>M 71</td>
<td>extensor tendon</td>
<td>134</td>
<td>+ +</td>
<td>+ -</td>
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<tr>
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<td>M 76</td>
<td>hip</td>
<td>145</td>
<td>+</td>
<td>- + -</td>
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<td>+</td>
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<td>F 62</td>
<td>wrist</td>
<td>157</td>
<td>+</td>
<td>- - -</td>
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<td>F 72</td>
<td>knee</td>
<td>190</td>
<td>+</td>
<td>- - -</td>
</tr>
<tr>
<td>332</td>
<td>MNK</td>
<td>NK</td>
<td>332</td>
<td>+</td>
<td>+ + -</td>
</tr>
<tr>
<td>385</td>
<td>F 54</td>
<td>hip</td>
<td>385</td>
<td>+</td>
<td>- - +</td>
</tr>
</tbody>
</table>

Key: + = present; - = absent; NK = not known

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Figure 3.2. Terminal GlcNAc expression in rheumatoid synovium. Widespread cellular and extracellular (arrow) terminal GlcNAc is seen. (Peroxidase developed with diaminobenzidine with haematoxylin counterstain × 20)
3.2.4. Expression of terminal GlcNAc on synovial cell nuclei

Normal (n=5) and rheumatoid (n=5) synovial samples were assessed for expression of terminal GlcNAc on cell nuclei using Mab GN7.

Normal synovium (Figure 3.3(a).) showed dense staining of the nuclei alone of the lining cells and scattered perivascular cells. All other cells showed barely detectable staining of the nucleus. Nuclei of lining cells were large and oval in contrast to nuclei of deep cells which were elongated and flattened. Rheumatoid synovium (Figure 3.3(b).) showed variable, moderately intense granular nuclear staining to be present on approximately 90% of cells, of a variety of types, situated in both superficial and deep layers of the tissue. Very few cells showed the faint staining seen in the deep layers of normal synovium. High-power fluorescence microscopy revealed a ring pattern of staining, suggesting binding of GN7 to the nuclear membrane (see Figure 3.10).

Figure 3.3. Expression of terminal GlcNAc on synovial cell nuclei. (a) Normal synovium shows nuclear terminal GlcNAc restricted to the lining layer. (b) Rheumatoid synovium shows widespread nuclear terminal GlcNAc. (Peroxidase developed with diaminobenzidine without counterstain x 20)
3.2.5. Confirmation of specificity of monoclonal antibody GN7 for terminal GlcNAc

The specificity of Mab GN7 for terminal GlcNAc was investigated on tissue by staining serial sections with GN7 and the plant lectin *Bandeiraea simplicifolia* GSII.

Normal (n=2) and rheumatoid (n=3) synovia all showed GN7 and Bandeiraea to give similar distributions of stain (Figure 3.4.).

![Figure 3.4](image)

Figure 3.4. Specificity of Mab GN7 for terminal GlcNAc compared to Bandeiraea. Rheumatoid synovium stained for terminal GlcNAc using (a) Mab GN7 and (b) Bandeiraea. Similar patterns of staining are seen. (Peroxidase developed with diaminobenzidine with haematoxylin counterstain x 20)

3.2.6. Conclusions of 3.2.

The specificity of Mab GN7 for terminal GlcNAc was confirmed. In normal synovium, histochemical studies using Mab GN7 showed terminal GlcNAc to be restricted to cell nuclei. Synovial lining cell nuclei were sharply distinguished from those of deep cells by their larger size, oval shape and a much greater intensity of expression of terminal GlcNAc. Rheumatoid synovial cells showed no such variation with almost all cells showing large rounded nuclei with a moderate
intensity of expression, regardless of site. In addition, all rheumatoid synovia showed terminal GlcNAc to be present in the cytoplasmic/membrane compartment of giant cells, haemosiderin-laden macrophages and a proportion of large, mononuclear cells distributed throughout the tissue. Eight of 10 rheumatoid samples also showed extracellular matrix terminal GlcNAc patchily distributed in two distinct patterns: fine, fibrillar and coarse reticular.

3.3. CYTOPLASMIC/MEMBRANE EXPRESSION OF TERMINAL GlcNAc IN RHEUMATOID SYNOVIAL

3.3.1. Introduction

Many cell types are represented in synovium and the aim of this section was to determine whether cytoplasmic/membrane terminal GlcNAc expression was restricted to a particular cell type. The distribution of GlcNAc-bearing cells in tissue and their morphology (see 3.2.3.) allowed lymphocytes to be excluded. Similar considerations excluded cells comprising blood vessels, lymphatics and nerve fibres. Macrophages, dendritic cells (Figure 3.5.), fibroblasts and mast cells remained as candidates.

Figure 3.5. GlcNAc+ rheumatoid synovial cell with dendritic morphology. (Peroxidase developed with diaminobenzidine with haematoxylin counterstain x 40)
A newly-available macrophage marker, CD68 (recognised by Mab EBM11) was assessed against the recognised macrophage markers, non-specific esterase activity and Mab RFD7. Using this marker in conjunction with Mab RFD1, a method of identifying dendritic cells was proposed. Terminal GlcNAc expression on macrophages and dendritic cells was then investigated in a series of double labelling experiments.

3.3.2. Markers of tissue macrophages

The suitability for use in synovium of the newly-available macrophage marker, CD68 (recognised by Mab EBM11) was assessed against the recognised markers, non-specific esterase activity and Mab RFD7.

3.3.2.1. CD68 compared to non-specific esterase activity in rheumatoid synovium

Samples of normal (n=3) and rheumatoid (n=5) synovium were tested for non-specific esterase activity and the same sections incubated with Mab EBM11. Normal tonsil served as a positive control. Cells positive for each marker alone and for both markers were counted. Cells only from the lining layer to a maximum depth of four cell widths were counted, in order to minimise inclusion of non-specific esterase-positive non-macrophages, such as B-lymphocytes and high endothelial cells.

A very small minority (1-14%) of cells were positive for non-specific esterase alone whereas a significant proportion (27-55%) of cells were positive for CD68 alone (Table 3.2.). The findings were similar in both normal and rheumatoid samples. In all samples, non-specific esterase activity was present in the bulk of the perinuclear cytoplasm only, whereas CD68 was also present at the periphery, including on cytoplasmic processes (Figure 3.6.).
Table 3.2.

THE DISTRIBUTION IN NORMAL AND RHEUMATOID SYNOVIAL LINING OF CD68 IN RELATION TO NON-SPECIFIC ESTERASE ACTIVITY

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>% OF CELLS</th>
<th>% OF CELLS</th>
<th>% OF CELLS</th>
<th>TOTAL CELLS COUNTED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD68+/NSE-</td>
<td>CD68+/NSE+</td>
<td>CD68-/NSE+</td>
<td></td>
</tr>
<tr>
<td>436 N</td>
<td>41</td>
<td>5</td>
<td>54</td>
<td>111</td>
</tr>
<tr>
<td>302 N</td>
<td>52</td>
<td>4</td>
<td>44</td>
<td>113</td>
</tr>
<tr>
<td>501 N</td>
<td>34</td>
<td>1</td>
<td>65</td>
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</tr>
<tr>
<td>506 RA</td>
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<td>6</td>
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<tr>
<td>431 RA</td>
<td>55</td>
<td>14</td>
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<td>114</td>
</tr>
</tbody>
</table>

Key: NSE = non-specific esterase activity; N = normal synovium; RA = rheumatoid synovium

Figure 3.6. CD68 compared to non-specific esterase activity in rheumatoid synovium. CD68+/NSE- (arrow) and CD68+/NSE+ (double arrow) cells are seen. No clearly CD68-/NSE+ cells are seen in this view. (Alkaline phosphatase-anti-alkaline phosphatase developed with fast blue without counterstain x 20)
3.3.2.2. CD68 compared to monoclonal antibody RFD7 in rheumatoid synovium

Samples of normal (n=3) and rheumatoid (n=5) synovium were incubated with Mabs EBM11 and RFD7 using a double immunofluorescence technique modified for use with two Mabs of the same immunoglobulin class. Cells positive for each marker alone and for both were counted. Cells only from the lining layer to a maximum depth of four cell widths were counted.

Virtually no cells were positive for RFD7 alone but a significant proportion (16-61%) were positive for CD68 alone (Table 3.3.). Findings were similar in both normal and rheumatoid samples. All samples showed RFD7 binding largely to only part of the cytoplasm of the cell body whereas CD68 was present throughout the cytoplasm, including cytoplasmic processes (Figure 3.7.).

Figure 3.7. CD68 compared to RFD7 positivity in rheumatoid synovium. Numerous CD68+/RFD7+ (yellow) (arrow) cells and CD68+/RFD7- (green) (double arrow) cells but few CD68-/RFD7+ (red) cells can be seen. (Indirect fluorescence x 20)
### Table 3.3.

**THE DISTRIBUTION IN NORMAL AND RHEUMATOID SYNOVIAL LINING OF CD68 IN RELATION TO RFD7**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>% OF CELLS CD68+/RFD7-</th>
<th>% OF CELLS CD68-/RFD7+</th>
<th>% OF CELLS CD68+/RFD7+</th>
<th>TOTAL CELLS COUNTED</th>
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<tbody>
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<td>501 N</td>
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<tr>
<td>495 RA</td>
<td>16</td>
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<td>102</td>
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</table>

Key: N = normal synovium; RA = rheumatoid synovium

#### 3.3.2.3. Summary and conclusions of 3.3.2.

CD68 is clearly superior to both non-specific esterase activity and Mab RFD7 as an identification marker of macrophages in synovium as it is more inclusive. Unlike non-specific esterase activity and the epitope of RFD7, it is densely present throughout the cytoplasm and thus gives a more accurate impression of overall shape and size.

#### 3.3.3. Relationship of terminal GlcNAc to CD68

Synovial tissues of patients (n=5) with rheumatoid arthritis were enzymatically disaggregated and cytospins of the resultant cell suspension incubated with Mabs
GN7 and EBM11. Binding was visualised by a double immunofluorescence technique for Mabs of differing immunoglobulin class. Cells whose cytoplasm labelled with each Mab were counted and assessed for double labelling.

Of approximately 300 cells counted in each sample (Table 3.4.), the proportion of GlcNAc+ cells which were also CD68+ varied widely - from 27% to 100% - and these cells formed a minority (9-18%) of the total population of CD68+ cells. Interestingly, many cells judged CD68+/GlcNAc - showed small, faintly GlcNAc+ granules scattered throughout the cytoplasm (Figure 3.8.).

Table 3.4.

THE DISTRIBUTION IN DISAGGREGATED RHEUMATOID SYNOVIAL CELLS OF TERMINAL GlcNAc IN RELATION TO THE MACROPHAGE Marker CD68

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>NUMBER OF CELLS CD68+</th>
<th>NUMBER OF CELLS GlcNAc+</th>
<th>% OF CELLS GlcNAc+ ALSO CD68+</th>
<th>% OF CELLS CD68+ ALSO GlcNAc+</th>
<th>TOTAL CELLS COUNTED</th>
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79
3.3.4. Dendritic cells

Previous studies have used Mab RFD1 in conjunction with the macrophage marker Mab RFD7 to identify dendritic cells in tissue. In these studies, RFD7 showed no overlap with RFD1. The discovery of CD68 (as recognised by Mab EBM11) has, however, demonstrated (see 3.3.2.2.) RFD7 to be unsatisfactory as a pan-macrophage marker. The specificity of RFD1 was therefore reassessed using anti-CD68 in double-labelling studies on rheumatoid synovium and on cells disaggregated from rheumatoid synovium.

3.3.4.1. Relationship between monoclonal antibody RFD1 and CD68 in rheumatoid synovial tissue

Samples of rheumatoid synovium (n=9) were incubated with RFD1 and EBM11 and binding visualised by an immunofluorescence technique for double-labelling with Mabs of differing immunoglobulin class.
RFD1+ cells were found in all samples, although in variable numbers, and were present in the lining layer, in the subintima and within lymphocyte aggregates (where these were present) (Table 3.5.). The proportion of RFD1+ cells which were also CD68+ varied with site within the tissue. Within the synovial lining, most RFD1+ cells expressed CD68 but within lymphocyte clusters very few did so. The RFD1+/CD68+ cells within the synovial lining were of rounded shape with a small number of cytoplasmic processes. In contrast, the RFD1+/CD68- cells within lymphocyte aggregates showed multiple branching processes closely apposed to the surrounding lymphocytes.

Table 3.5.

THE MEAN (RANGE) PERCENTAGE OF RFD1+ CELLS ALSO CD68+ IN DIFFERENT AREAS OF RHEUMATOID SYNOVIIUM (n=9)

<table>
<thead>
<tr>
<th>Area</th>
<th>Percentage</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial Lining</td>
<td>89.5</td>
<td>0-100</td>
</tr>
<tr>
<td>Subintima</td>
<td>41.3</td>
<td>0-100</td>
</tr>
<tr>
<td>Lymphocyte Aggregates</td>
<td>6.3</td>
<td>0-33</td>
</tr>
</tbody>
</table>

3.3.4.2. Relationship between monoclonal antibody RFD1 and CD68 in cells disaggregated from rheumatoid synovium

Samples of rheumatoid synovium from different patients (n=4) were enzymatically disaggregated and cytospins of the resultant cell suspension incubated with Mabs RFD1 and EBM11. Binding was visualised by a double immunofluorescence technique adapted for use with two Mabs of differing immunoglobulin class. Cells whose cytoplasm labelled with each Mab were counted.
and assessed for double labelling.

Although information regarding site in the tissue is lost in this experiment, double-labelling may be more accurately assessed on single cells (Figure 3.9.) and it is clear that of the total number of RFD1+ cells only a minority (4-26%) are also CD68-. (Table 3.6.).

Table 3.6.

THE DISTRIBUTION IN DISAGGREGATED RHEUMATOID SYNOVIAL CELLS OF THE DENDRITIC CELL MARKER RFD1 IN RELATION TO THE MACROPHAGE MARKER CD68

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>NUMBER OF CELLS CD68+</th>
<th>NUMBER OF CELLS RFD1+</th>
<th>% OF CELLS RFD1+ ALSO CD68+</th>
<th>% OF CELLS RFD1+ ALSO CD68-</th>
<th>TOTAL CELLS COUNTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>105</td>
<td>54</td>
<td>96</td>
<td>4</td>
<td>305</td>
</tr>
<tr>
<td>2</td>
<td>118</td>
<td>35</td>
<td>77</td>
<td>23</td>
<td>314</td>
</tr>
<tr>
<td>3</td>
<td>135</td>
<td>41</td>
<td>90</td>
<td>10</td>
<td>323</td>
</tr>
<tr>
<td>4</td>
<td>81</td>
<td>27</td>
<td>74</td>
<td>26</td>
<td>307</td>
</tr>
</tbody>
</table>

3.3.4.3. Summary and conclusions of 3.3.4.

RFD1 binding clearly overlaps with CD68 expression but in areas of rheumatoid synovium where dendritic cells would be expected, i.e. within lymphocyte clusters, the percentage of overlap is very low. Moreover, RFD1+/CD68- cells in these areas have the morphology expected of dendritic cells. It thus seems reasonable to propose that RFD1 positivity in conjunction with CD68 negativity identifies
antigen-presenting dendritic cells more accurately than RFD1 positivity alone.

Figure 3.9. Relationship between CD68 and RFD1 positivity in rheumatoid synovial cells. A single RFD1+/CD68- cell (red) is seen, RFD1-/CD68+ cells (green), several of which show a blush of RFD1 positivity (yellow) (arrow). (Indirect fluorescence x 40)

3.3.5. Relationship of terminal GlcNAc to RFD1 positivity

Synovial tissues from patients (n=5) with RA were enzymatically disaggregated and cytospins of the resultant cell suspension incubated with Mabs GN7 and RFD1. Binding was visualised by a double immunofluorescence technique modified for use with two Mabs of the same immunoglobulin class. Cells whose cytoplasm labelled with each Mab were counted and assessed for double labelling.

Of approximately 300 cells counted in each sample (Table 3.7.), the proportion of GlcNAc+ cells which were also RFD1+ varied from 13% to 56%. The proportion of RFD1+ cells which were also GlcNAc+ varied from 7% to 45%. The degree of double labelling of individual cells, as judged by the final colour of fluorescence, varied markedly, from yellowish-green, through yellow to orange-red (Figure 3.10.). Only yellow cells were judged to be RFD1+/GlcNAc+.
Table 3.7.

THE DISTRIBUTION IN DISAGGREGATED RHEUMATOID SYNOVIAL CELLS
OF TERMINAL GlcNAc IN RELATION TO THE DENDRITIC CELL MARKER
RFD1

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>NUMBER OF CELLS</th>
<th>NUMBER OF RFD1+ CELLS</th>
<th>GlcNAc+ CELLS</th>
<th>GlcNAc+ ALSO RFD1+ CELLS</th>
<th>GlcNAc+ COUNTED</th>
<th>% OF CELLS GlcNAc+</th>
<th>% OF CELLS RFD1+</th>
<th>TOTAL CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>8</td>
<td>50</td>
<td>10</td>
<td>312</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>55</td>
<td>13</td>
<td>20</td>
<td>315</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>18</td>
<td>50</td>
<td>13</td>
<td>317</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>16</td>
<td>56</td>
<td>45</td>
<td>318</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>55</td>
<td>11</td>
<td>36</td>
<td>7</td>
<td>333</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.10. Relationship between terminal GlcNAc and RFD1 positivity in rheumatoid synovial cells. Cells judged to be GlcNAc+/RFD1- (red and orange-red), GlcNAc-/RFD1+ (green and yellow-green) and GlcNAc/RFD1+ (yellow) are seen. Terminal GlcNAc is especially prominent on cell nuclei (arrow) (see 3.2.4.) in this view. (Indirect fluorescence x 40)
3.3.6. Conclusions of 3.3.

CD68 has been shown to be superior to non-specific esterase activity and Mab RFD7 as a marker of synovial macrophages. Double labelling histochemical techniques showed that a small (9-18%) proportion of synovial macrophages expressed terminal GlcNAc and also that only a proportion (27-100%) of GlcNAc+ cells expressed CD68. That is, the two markers overlapped but did not codistribute.

Dendritic cells have been shown to require a double label (RFD1+/CD68-) for reliable identification. Given that GN7, EBM11 and RFD1 are all mouse Mabs, a specific relationship between terminal GlcNAc and dendritic cells could not be established as, without facilities for triple immunofluorescence, it was not possible to triple-label reliably with antibodies of the same species of origin. The possibility that all RFD1+/CD68- cells were also GlcNAc+ could not therefore be excluded. However, only a proportion (13-56%) of GlcNac+ cells were also RFD1+, indicating that terminal GlcNAc expression was not restricted to RFD1+ cells.

The distribution of terminal GlcNAc overlapped with both CD68 and the RFD1 epitope but did not coincide with either. This indicated that terminal GlcNAc could be expressed by at least two distinct cell types, but by only a proportion of the cells of each type, and therefore did not designate a particular cell type. Further double-labelling experiments to investigate fibroblasts and mast cells were therefore not performed.
3.4. EXTRACELLULAR EXPRESSION OF TERMINAL GlcNAc IN RHEUMATOID SYNOVIA

3.4.1. Introduction

Terminal GlcNAc present in the extracellular space of rheumatoid synovium was investigated in terms of its distribution in relation to structural features of the tissue and in relation to the distribution of certain common glycosylated molecules. Hyaluronan (a glycosaminoglycan) and fibronectin (a glycoprotein) are structural molecules of the extracellular matrix. Fibrin and immunoglobulin G (both glycoproteins) are deposited in the extracellular space from the interstitial fluid.

3.4.2. Distribution of extracellular terminal GlcNAc and its relation to structural features

Samples of rheumatoid synovium (n=11) were preselected for the presence of extracellular GlcNAc and incubated with Mab GN7. Serial sections were double-labelled with GN7 and Ulex to identify blood vessels. The distribution of extracellular GlcNAc was assessed in relation to the following structural features of the tissue: lining layer, deep layers, blood vessels, lymphoid aggregates and tissue splitting (Table 3.8.). Extracellular GlcNAc was deemed to be associated with a structural feature if the two were seen associated at at least one site.

Coarse reticular GlcNAc was present on parts of the tissue surface (Figure 3.11(a).) in all samples and in the deep layers in seven, where it was constantly associated with tissue splitting. Two samples contained fine fibrillar GlcNAc distributed around small blood vessels (Figure 3.11(b).). Neither type of GlcNAc was found in association with lymphoid aggregates.
Table 3.8.

EXTRACELLULAR TERMINAL GlcNAc DISTRIBUTION IN RHEUMATOID SYNOVIUM IN RELATION TO STRUCTURAL FEATURES OF THE TISSUE

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PATTERN OF GlcNAc</th>
<th>SITE</th>
<th>BLOOD VESSELS</th>
<th>LYMPHOID AGGREGATES</th>
<th>TISSUE SPLITTING</th>
</tr>
</thead>
<tbody>
<tr>
<td>138</td>
<td>OR</td>
<td>surface</td>
<td>-</td>
<td>P -</td>
<td>A</td>
</tr>
<tr>
<td>139</td>
<td>OR</td>
<td>surface, deep</td>
<td>-</td>
<td>P -</td>
<td>P +</td>
</tr>
<tr>
<td>332</td>
<td>OR</td>
<td>surface, deep</td>
<td>-</td>
<td>P -</td>
<td>P +</td>
</tr>
<tr>
<td></td>
<td>FF</td>
<td>deep</td>
<td>P +</td>
<td>P -</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>OR</td>
<td>surface, deep</td>
<td>-</td>
<td>P -</td>
<td>P +</td>
</tr>
<tr>
<td></td>
<td>FF</td>
<td>deep</td>
<td>P +</td>
<td>P -</td>
<td></td>
</tr>
<tr>
<td>158</td>
<td>OR</td>
<td>surface, deep</td>
<td>-</td>
<td>A</td>
<td>P +</td>
</tr>
<tr>
<td>432</td>
<td>OR</td>
<td>surface</td>
<td>-</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>167</td>
<td>OR</td>
<td>surface, deep</td>
<td>-</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>197</td>
<td>OR</td>
<td>surface, deep</td>
<td>-</td>
<td>P -</td>
<td>P +</td>
</tr>
<tr>
<td>189</td>
<td>OR</td>
<td>surface, deep</td>
<td>-</td>
<td>P -</td>
<td>P +</td>
</tr>
<tr>
<td>188</td>
<td>OR</td>
<td>surface, deep</td>
<td>-</td>
<td>A</td>
<td>P +</td>
</tr>
<tr>
<td>168</td>
<td>OR</td>
<td>surface</td>
<td>-</td>
<td>P -</td>
<td>P -</td>
</tr>
</tbody>
</table>

Key: OR = coarse reticular extracellular GlcNAc; FF = fine fibrillar extracellular GlcNAc; P = structural feature present; A = structural feature absent; + = associated extracellular GlcNAc; - = no associated extracellular GlcNAc
Figure 3.11. Distribution of extracellular terminal GlcNAc in rheumatoid synovium. (a) Coarse reticular terminal GlcNAc is seen at the tissue surface in association with fibrin (arrow). (b) Fine fibrillar terminal GlcNAc (arrow) is seen in relation to blood vessels. (Alkaline phosphatase-anti-alkaline phosphatase developed with fast red with haematoxylin counterstain (a) x 10, (b) x 20)

3.4.3. Morphological relationship between extracellular terminal GlcNAc and glycosylated molecules present in the extracellular space

Serial sections of samples (n=4) of rheumatoid synovium selected for the presence of extracellular GlcNAc were incubated with Mab GN7, rabbit antibodies to human IgG, fibrin/fibrinogen and fibronectin, and with the binding region probe for hyaluronan. Serial sections of each sample were compared for codistribution of stain.

IgG, fibronectin and hyaluronan were each distributed extensively throughout all tissues but none codistributed with GlcNAc. (For a detailed treatment of the relationship between GlcNAc and hyaluronan, see 3.6.) Fibrin, however, showed a complex relationship with GlcNAc (Table 3.9.). Coarse reticular GlcNAc was often
found in a band within the tissue immediately underlying surface fibrin (see Figure 3.11(a).) and the margins of the fibrin deposits were often densely outlined with GlcNAc. Areas of deep tissue splitting also showed fibrin deposits at the site of split with underlying coarse reticular GlcNAc. None of these associations was, however, constant and whereas in these tissues coarse reticular extracellular GlcNAc was present only in areas of fibrin deposition, not all areas of fibrin deposition were associated with GlcNAc.

Table 3.9.

CODISTRIBUTION OF FIBRIN AND EXTRACELLULAR TERMINAL GlcNAc IN RHEUMATOID SYNOVUM

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>SEX AND AGE (yr)</th>
<th>SITE</th>
<th>LOCATION OF FIBRIN WITHIN TISSUE</th>
<th>ASSOCIATED GlcNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>431</td>
<td>F 54 knee</td>
<td></td>
<td>surface deep splitting (patchy)</td>
<td>+</td>
</tr>
<tr>
<td>438</td>
<td>F 62 hip</td>
<td></td>
<td>surface (patchy)</td>
<td>+</td>
</tr>
<tr>
<td>356</td>
<td>F 75 knee</td>
<td></td>
<td>surface deep splitting</td>
<td>+</td>
</tr>
<tr>
<td>476</td>
<td>F 50 hip</td>
<td></td>
<td>surface</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: + = present

3.4.4. Conclusions of 3.4.

In rheumatoid synovium, fine fibrillar extracellular GlcNAc was rare but when present was found in patches in the deep layers occasionally related to blood vessels. Coarse reticular GlcNAc was the commoner pattern and was found related
to fibrin deposition both on the tissue surface and at sites of tissue splitting. There was no relation to the distribution of other glycosylated molecules such as hyaluronan, fibronectin and immunoglobulin G.

3.5. SPECIFICITY OF CHANGES IN TERMINAL GlcNAc EXPRESSION FOR RHEUMATOID SYNOVITIS

3.5.1. Introduction

Inflamed non-synovial tissues and synovium affected by non-rheumatoid disease were investigated for terminal GlcNAc expression in order to guard against inappropriate conclusions regarding the specificity of the features observed above for rheumatoid arthritis.

3.5.2. Terminal GlcNAc expression in non-rheumatoid synovial disease

Samples (n=4) of synovium from patients with osteoarthritis were incubated with Mab GN7.

Two samples showed cellular and extracellular staining indistinguishable from that seen in rheumatoid synovium (Figure 3.12.). Both samples, unlike the GlcNAc-

Figure 3.12. Terminal GlcNAc expression in osteoarthritic synovium. Extracellular terminal GlcNAc of coarse reticular pattern is seen. (Peroxidase developed with diaminobenzidine without counterstain x 20.)
samples, showed features of inflammation such as increased cellularity of the lining layer and a moderate mononuclear cell infiltrate.

3.5.3 Terminal GlcNAc expression in inflammatory disease in non-synovial tissues

Miscellaneous non-synovial tissues affected by inflammatory disease of varying aetiology were incubated with Mab GN7. The tissues investigated were: inflamed anal skin overlying prolapsed haemorrhoids (n=1), dehisced surgical wound (n=1), wall of gall bladder affected by cholelithiasis (n=2), wall of colon affected by ulcerative colitis (n=1), wall of tuberculous abscess (n=2).

All samples except gall bladder wall showed extracellular terminal GlcNAc; cell-associated terminal GlcNAc was present in gall bladder wall and tuberculous abscess wall (Figure 3.13.).

Figure 3.13. Terminal GlcNAc expression in tuberculous abscess wall. (a) Coarse reticular extracellular terminal GlcNAc is seen. (b) Scattered GlcNAc+ mononuclear cells are seen. (Peroxidase developed with ethylaminocarbazole with haematoxylin counterstain x 20)
3.5.4. Conclusions of 3.5.

Neither cellular nor extracellular terminal GlcNAc is specific for rheumatoid synovitis but rather appear to be features of inflammation per se.

3.6. RELATION BETWEEN TERMINAL GlcNAc AND HYALURONAN

3.6.1. Introduction

Terminal GlcNAc as identified by Mab GN7 does not co-distribute with hyaluronan as identified by the binding region probe (see 3.4.3.). As hyaluronan chains comprise repeating disaccharide units, each of which contains GlcNAc and glucuronic acid, each hyaluronan molecule must bear one terminal GlcNAc moiety. Native hyaluronan molecules are of very high molecular weight so a given mass or volume may contain few terminal GlcNAc elements. Binding of Mab GN7 to high-molecular weight, native hyaluronan was assessed by the method of dot blotting.

The binding region probe requires a complete, accessible decasaccharide length of the hyaluronan molecule for binding and clearly will not bind to very small fragments. Mab GN7 may, however, be preferentially distributed to small hyaluronan fragments which, for a given volume or mass, will bear proportionately more terminal GlcNAc elements. Streptomyces hyaluronidase cleaves hyaluronan down to its constituent disaccharides, most of which are then probably lost from the tissue in washings. The effect of prior Streptomyces hyaluronidase digestion on the binding of Mab GN7 to tissue was assessed. Binding of Mab GN7 to low-molecular weight oligosaccharides of hyaluronan was assessed by the method of dot blotting.

3.6.2. Binding of monoclonal antibody GN7 to hyaluronan as assessed by dot blotting

Native, high-molecular weight hyaluronan and hyaluronan oligosaccharides were
applied to strips of nitrocellulose paper. Chondroitin sulphate, which does not contain GlcNAc, was used as a control. Separate strips were incubated with Mab GN7 and the binding region probe for hyaluronan. Binding was visualised by a peroxidase system.

The binding region probe bound to native hyaluronan bound to the nitrocellulose but Mab GN7 showed no binding (Figure 3.14.). Neither the probe nor Mab GN7 showed binding to the sites on the nitrocellulose of application of the oligosaccharides or chondroitin sulphate. The experiment was repeated three times with similar results.

![Figure 3.14. Binding of Mab GN7 to hyaluronan as assessed by dot blotting. The binding region probe bound to native hyaluronan but no binding was seen to hyaluronan oligosaccharides or chondroitin sulphate. Mab GN7 showed no binding. (Peroxidase developed with diaminobenzidine.)](image)

3.6.3. Effect of pre-treatment of tissues with *Streptomyces* hyaluronidase on binding of monoclonal antibody GN7

Samples of rheumatoid synovium (n=2), normal skin (n=2) and human umbilical
cord (n=1) were pretreated with Streptomyces hyaluronidase and then incubated with Mab GN7. Serial sections not treated with the enzyme served as controls. Comparison of experimental with control sections showed no difference in the distribution or intensity of staining for terminal GlcNAc (Figure 3.15.).

![Figure 3.15. Effect of prior hyaluronidase digestion on Mab GN7 binding to rheumatoid synovium. (a) Hyaluronidase-treated tissue (b) Control. Staining is unaffected. (Peroxidase developed with ethylaminocarbazole with haematoxylin counterstain x 10)](image)

Dot blotting showed no detectable binding of Mab GN7 to native hyaluronan or to hyaluronan oligosaccharides. The binding region probe for hyaluronan showed binding to native hyaluronan but, surprisingly, no detectable binding to oligosaccharides. Oligosaccharides from the same batch were later demonstrated (see 4.2.3.) to bind the binding region probe in solution so failure to demonstrate binding of the probe to oligosaccharides in a dot blotting experiment suggested that the oligosaccharides had failed to bind to the nitrocellulose. Hence, no conclusions regarding binding of GN7 to oligosaccharides could be drawn. No explanation was
found in this study for the failure of hyaluronan oligosaccharides to bind to nitrocellulose.

Streptomyces hyaluronidase digestion of tissues known to exhibit GN7 binding and to contain hyaluronan had no effect on the intensity or distribution of GN7 binding. This strongly suggested that extracellular terminal GlcNAc, in the configuration recognised by Mab GN7, is not carried on hyaluronan.

3.7. OVERALL CONCLUSIONS OF CHAPTER 3

Using histochemical techniques and the specific Mab GN7, terminal GlcNAc expression was shown to be upregulated in diseased tissue compared to normal but this increase in expression was found not to be specific to rheumatoid disease or to synovium. Terminal GlcNAc was found in osteoarthritic synovium and in a variety of non-synovial tissues affected by inflammation due to autoimmune disease, infection and mechanical irritation.

Terminal GlcNAc was found to be constitutively expressed on synovial cell nuclear membrane but the degree of expression (as judged by the intensity of staining with Mab GN7) varied, being greatest on cells of the normal synovial lining and barely detectable on cells of the deep layers. This finding provided further evidence for the specialised nature of these cells. Rheumatoid synovial cells showed no such differentiation of the superficial layer; a moderate degree of expression of nuclear terminal GlcNAc was present throughout the tissue.

In diseased tissue, terminal GlcNAc was present in the cytoplasmic/membrane compartment of a proportion of large mononuclear cells. In rheumatoid synovium, cytoplasmic/membrane GlcNAc was present on a number of different cell types. A minority of macrophages and RFD1+ cells expressed GlcNAc at any one time, as did
a proportion of non-macrophages (which in these experiments were probably mainly fibroblasts and a small number of endothelial cells). Cytoplasmic/membrane GlcNAc did not, therefore, characterise any single cell type. During these studies, CD68 was assessed against and found to be superior to both non-specific esterase and the epitope of Mab RFD7 as a pan-macrophage marker in synovium. Using CD68, the specificity of Mab RFD1 for dendritic cells was reassessed and a new criterion (CD68⁻/RFD1+) proposed for identification of dendritic cells in synovium.

Terminal GlcNAc was found to be present in the extracellular space in diseased tissue. In rheumatoid synovium, extracellular GlcNAc of a coarse reticular pattern was related to fibrin deposition, whether on the tissue surface or at the site of tissue splitting, rather than to any other structural feature. (A minority of tissues also contained small areas of GlcNAc of fine fibrillar pattern in relation to small blood vessels.) Despite the presence of GlcNAc on one end of each molecule of hyaluronan, Mab GN7 binding to tissue appeared unrelated to hyaluronan, whether native or degraded.

Terminal GlcNAc appeared to be a non-specific tissue marker of inflammatory disease expressed in synovium by several cell types and, in the extracellular space, related to fibrin.
4. RESULTS II: MORPHOLOGICAL DISTRIBUTION OF THE GLYCOSAMINOGLYCANS, HYALURONAN, CHONDROITIN-4-SULPHATE/DERMATAN SULPHATE (Ch4S/DS) AND CHONDROITIN-6-SULPHATE (Ch6S), IN TISSUE AND ALTERATION IN INFLAMMATORY DISEASE

4.1. GENERAL INTRODUCTION

The binding region probe for hyaluronan was assessed for its suitability as a histochemical marker of hyaluronan in synovium. Conditions of staining yielding optimal results were determined and the specificity of the probe for hyaluronan was investigated. Monoclonal antibody (Mab) NDOG1, a putative anti-hyaluronan reagent, was compared with the probe as a marker of hyaluronan in tissue sections. Using the binding region probe, the morphological distribution of hyaluronan in normal and diseased synovium and its relation to the cellular elements in rheumatoid synovium were investigated. The closely-related glycosaminoglycans, Ch4S/DS and Ch6S, were mapped in normal and diseased synovium using Mabs and the distribution of all three glycosaminoglycans, Ch4S/DS, Ch6S and hyaluronan, was compared in a variety of normal non-synovial tissues and their diseased counterparts. A brief study was made of the histological expression of the extracellular receptor CD44 which is known to bind to hyaluronan and chondroitin sulphate.

4.2. PROBLEMS AND DEVELOPMENTS IN ASSESSING THE MORPHOLOGICAL DISTRIBUTION OF HYALURONAN IN TISSUE

4.2.1. Introduction

Methods of optimising conditions of staining with the binding region probe on synovium and its specificity for hyaluronan were investigated. A putative hyaluronan-binding reagent, Mab NDOG1, was assessed in comparison with the binding region probe on tissues known to be rich in hyaluronan.
4.2.2. Use of the binding region probe to identify hyaluronan in synovium

The traditional fixative for hyaluronan, cetylpyridinium chloride, was assessed for its suitability for use on inflamed synovium. A range of fixatives containing various concentrations of cetylpyridinium chloride were assessed for their suitability for double labelling with the binding region probe and Mabs. This was necessary as Mab epitopes can be sensitive to certain forms of fixation. A time course was performed to establish the length of incubation required for optimal staining of tissue by the binding region probe.

4.2.2.1. Cetylpyridinium chloride as fixative

Serial samples of rheumatoid synovium were fixed in cetylpyridinium chloride with prior washing in PBS for 0, 1, 5, or 30 minutes and then incubated with the binding region probe.

Samples fixed in cetylpyridinium chloride without prior rinsing showed densely stained accretions scattered randomly over the tissue and in a band along the tissue surface (Figure 4.1.). There was no staining of the underlying tissue. Samples...
fixed after rinsing in PBS for 5 or 30 minutes showed no accretions but widespread tissue staining as detailed below (section 4.3.3. and Figure 4.5.(a)). Samples fixed after rinsing for 1 minute showed small accretions but no tissue staining. Similar but unstained accretions were also visible as refractile bodies on control sections not exposed to the binding region probe when these were viewed by phase contrast microscopy.

4.2.2.2. Time course for incubation with the binding region probe
Serial samples of rheumatoid synovium were rinsed, fixed in cetylpyridinium chloride and incubated with the binding region probe for 1, 2, 3 and 5 hours and overnight (>8 hours).

Incubation for 1, 2 or 3 hours gave patchy, uneven staining which, although faint, was of increasing intensity with increasing duration of incubation. Incubation for 5 hours gave intense staining which was not increased in intensity by overnight incubation. Reagent controls were uniformly unstained.

4.2.2.3. Fixatives for double labelling with monoclonal antibodies and the binding region probe
Serial samples of rheumatoid synovium were exposed to a variety of fixatives (Table 4.1.) and then incubated with either the binding region probe or Mab EBM11.

All fixation methods involving cetylpyridinium chloride gave staining of impaired quality with Mab EBM11 when compared with acetone; that is, the staining was of reduced intensity and poorly demarcated. Methanol alone and acetone alone each gave staining with the binding region probe of similar distribution and intensity to that obtained with cetylpyridinium chloride fixation preceded by rinsing. Of the fixation methods assessed, only acetone gave acceptable staining with both the
binding region probe and Mab EBM11.

Table 4.1.

**ASSESSMENT OF FIXATIVES FOR SUITABILITY FOR DOUBLE-LABELLING WITH THE BINDING REGION PROBE AND MONOCLONAL ANTIBODIES**

<table>
<thead>
<tr>
<th>FIXATIVE</th>
<th>STAINING WITH BINDING REGION PROBE</th>
<th>STAINING WITH Mab EBM11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetone F/B 0.5% CPC in PBS*</td>
<td>+</td>
<td>+ / -</td>
</tr>
<tr>
<td>Acetone F/B 0.1% CPC in PBS*</td>
<td>+</td>
<td>+ / -</td>
</tr>
<tr>
<td>Acetone F/B 0.01% CPC in PBS*</td>
<td>+</td>
<td>+ / -</td>
</tr>
<tr>
<td>Methanol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glutaraldehyde with 0.5% CPC*</td>
<td>+</td>
<td>+ / -</td>
</tr>
<tr>
<td>Glutaraldehyde with 0.1% CPC*</td>
<td>+</td>
<td>+ / -</td>
</tr>
<tr>
<td>Glutaraldehyde with 0.01% CPC*</td>
<td>+</td>
<td>+ / -</td>
</tr>
</tbody>
</table>

Key: + = present; - = absent; +/- = impaired; F/B = followed by; CPC = cetyl pyridinium chloride; * = sections were rinsed in PBS for 5 minutes immediately prior to exposure to CPC

4.2.3. Confirmation of the specificity of the binding region probe for hyaluronan

Samples of normal skin (n=1), normal synovium (n=2) and rheumatoid synovium (n=2) were incubated with the binding region probe. Serial samples were digested with Streptomyces hyaluronidase prior to application of the probe. Further serial samples were incubated with aliquots of probe which had been incubated with either chondroitin sulphate or hyaluronan oligosaccharides.

Neither hyaluronidase-treated sections nor sections incubated with the probe in the presence of hyaluronan oligosaccharides showed staining. Staining was unaffected by the presence of chondroitin sulphate.
4.2.4. Comparison of monoclonal antibody NDOG1 with the binding region probe for identifying hyaluronan

The histochemical specificity for hyaluronan of Mab NDOG1 was investigated by comparing the pattern of staining obtained with this Mab with staining with the binding region probe, using tissues known to contain large amounts of hyaluronan. The effect on Mab staining of prior digestion of the tissues with Streptomyces hyaluronidase was assessed.

4.2.4.1. Comparison of staining with monoclonal antibody NDOG1 and the binding region probe.

Serial sections of normal skin (n=3), umbilical cord (n=1) and placenta (n=1) were incubated with the NDOG1 Mab and with the binding region probe.

The results were as follows:

(i) skin

With the binding region probe, samples showed dense staining of both epidermis and dermis with a condensation of stain around hair follicles, sweat glands and blood vessels (Figure 4.2(a)). With Mab NDOG1, the stain was distributed to the epidermis, hair follicles and sweat glands with faint staining of blood vessel walls. The dermis, however, apart from faint fibrillar staining around hair follicles and blood vessels, was unstained (Figure 4.2(b)).

(ii) umbilical cord

The binding region probe showed moderately intense staining of the limiting membrane of the umbilical cord with patchy dense staining of the blood vessel media, more marked in the two arteries than in the vein. There was faint staining throughout the matrix with a denser lamellar arrangement of stain superimposed. Mab NDOG1 showed a similar pattern of stain, although the intensity was greater, with the addition of staining of the smooth muscle cells of the blood vessel media.
The binding region probe and Mab NDOG1 showed identical patterns of staining. There was dense surface staining outlining the placental villi while the villous substance was unstained.

Figure 4.2. Binding region probe for hyaluronan compared to Mab NDOG1 on normal skin. (a) Binding region probe shows staining of the dermis and a band of stain around hair follicles. (b) Mab NDOG1 shows staining of hair follicle epithelium but no staining of dermis. (Alkaline phosphatase-anti-alkaline phosphatase developed with fast blue without counterstain x 20)

4.2.4.2. Effect of Streptomyces hyaluronidase on staining with monoclonal antibody NDOG1

Serial sections of skin, umbilical cord and placenta were incubated with Mab NDOG1 with and without prior digestion with Streptomyces hyaluronidase.

Hyaluronidase treatment left NDOG1 staining largely unaffected (Table 4.2., Figure 4.3.).
Table 4.2.

EFFECT OF HYALURONIDASE TREATMENT ON STAINING OF HYALURONAN-RICH TISSUES WITH Mab NDOG1

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>NDOG1 WITHOUT HYAL'ASE</th>
<th>NDOG1 WITH HYAL'ASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>epidermis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>hair follicle</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>blood vessel</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>sweat gland</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>dermal matrix (fibrillar</td>
<td>+</td>
<td>/ -</td>
</tr>
<tr>
<td>stain around dermal structures)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Umbilical cord</td>
<td></td>
<td></td>
</tr>
<tr>
<td>limiting membrane</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>matrix (lamellar staining)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>vascular SMCs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Placenta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>surface of villi</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: + = staining present; - = staining absent; +/- = staining present but reduced in intensity; SMCs = smooth muscle cells; hyal'ase = hyaluronidase

Figure 4.3. Effect of hyaluronidase on binding of Mab NDOG1 to normal skin. Staining is largely unaltered (compare with Figure 4.2(b).). (Alkaline phosphatase-anti-alkaline phosphatase developed with fast blue without counterstain x 20)
4.2.5. Conclusions of 4.2.

The binding region probe may be used successfully to visualise hyaluronan in frozen sections of synovium, both normal and diseased. Visualisation was optimised if tissues were fixed with either acetone or a fixative containing 0.5% cetylpyridinium chloride with prior washing of the sections in PBS for 5 minutes and if incubation times were 5 hours or greater. Cetylpyridinium chloride fixation without prior washing led to the appearance of scattered accretions, presumed to be precipitations of free, mobile or unbound hyaluronan. For double labelling with Mabs, acetone fixation was most suitable.

Using a number of methods, the binding region probe was shown to be specific for hyaluronan. Taking the binding region probe as the standard, Mab NDOG1 was shown to be low in both specificity and sensitivity as a probe for hyaluronan in tissue sections.

4.3. DISTRIBUTION OF HYALURONAN IN SYNOVIUM AND RELATION TO CELLULAR ELEMENTS

4.3.1. Introduction

The morphological distribution of hyaluronan in normal, rheumatoid and osteoarthritic synovium was investigated using the binding region probe. The relation of hyaluronan to macrophages (marked by CD68) and lymphocytes (marked by CD22 and CD3) in rheumatoid synovium was investigated, as was the distribution of cell-associated hyaluronan in cells enzymatically disaggregated from rheumatoid synovium.

4.3.2. Distribution of hyaluronan in normal synovium

Samples (n=5) of synovium obtained from normal joints were assessed for hyaluronan distribution using the binding region probe. Samples were taken from the tibio-talar joint (n=2), knee (n=2) and elbow (n=1) and no two samples were
taken from the same joint.

The samples showed varying morphology with intimal layers of one to six cells thickness and subintimal layers of fibrous, adipose and areolar type. There was no association between morphology and joint of origin. All samples, regardless of tissue architecture and cellularity, showed similar patterns of staining with the binding region probe. The tissue was stained throughout its depth but with much greater intensity in the lining layer (Figure 4.4). Lining layer staining showed a pericellular reticular pattern which was more pronounced in samples with a thicker, more cellular intima. In contrast, the faint generalised staining of the deeper layers was largely featureless. The walls of blood vessels of all sizes showed a condensation of stain in the media and adventitia but intima was unstained.

Figure 4.4 Distribution of hyaluronan in normal synovium. (Peroxidase developed with ethylaminocarbazole without counterstain x 10)

4.3.3. Distribution of hyaluronan in diseased synovium

Samples of rheumatoid (n=10) and osteoarthritic (n=5) synovium from various sites (Table 4.3.) were assessed for distribution of hyaluronan using the binding
Table 4.3.

PATIENT DETAILS AND SITE OF ORIGIN OF SYNOVUM

<table>
<thead>
<tr>
<th>AGE, yr</th>
<th>SEX</th>
<th>SITE</th>
<th>DISEASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>73</td>
<td>M</td>
<td>Extensor tendons</td>
<td>RA</td>
</tr>
<tr>
<td>66</td>
<td>F</td>
<td>Extensor tendons</td>
<td>RA</td>
</tr>
<tr>
<td>55</td>
<td>F</td>
<td>Elbow</td>
<td>RA</td>
</tr>
<tr>
<td>57</td>
<td>F</td>
<td>Elbow</td>
<td>RA</td>
</tr>
<tr>
<td>62</td>
<td>F</td>
<td>Wrist</td>
<td>RA</td>
</tr>
<tr>
<td>45</td>
<td>F</td>
<td>Wrist</td>
<td>RA</td>
</tr>
<tr>
<td>70</td>
<td>F</td>
<td>Knee</td>
<td>RA</td>
</tr>
<tr>
<td>61</td>
<td>F</td>
<td>Knee</td>
<td>RA</td>
</tr>
<tr>
<td>57</td>
<td>F</td>
<td>Knee</td>
<td>RA</td>
</tr>
<tr>
<td>56</td>
<td>F</td>
<td>Knee</td>
<td>RA</td>
</tr>
<tr>
<td>66</td>
<td>M</td>
<td>Hip</td>
<td>OA</td>
</tr>
<tr>
<td>50</td>
<td>F</td>
<td>Hip</td>
<td>OA</td>
</tr>
<tr>
<td>77</td>
<td>F</td>
<td>Hip</td>
<td>OA</td>
</tr>
<tr>
<td>67</td>
<td>M</td>
<td>Knee</td>
<td>OA</td>
</tr>
<tr>
<td>69</td>
<td>F</td>
<td>Knee</td>
<td>OA</td>
</tr>
</tbody>
</table>

Key: RA = rheumatoid arthritis; OA = osteoarthritis

Rheumatoid synovial samples showed intense staining throughout the tissue with slightly less intense staining of the hyperplastic lining layer (Figure 4.5(a)). Some samples showed a denser band of stain running immediately beneath the lining layer, in a region known to be populated by fibroblasts. A dense reticular pattern of stain outlined individual cells in areas of heavy cellular infiltration. Stain was present throughout the media and adventitia of large blood vessels and a narrow band of stain was present outside the capillary endothelial wall. In sparsely cellular, fibrotic areas staining was generally faint and of a fibrillar pattern.

Osteoarthritic synovia showed generalised fibrillar staining of low or moderate
intensity with an increase in intensity in the outer walls of blood vessels. Those specimens with areas of prominent inflammatory cell infiltrate showed a dense reticular pattern of stain around the infiltrating cells (Figure 4.5(b).), as seen in rheumatoid tissues. Most tissues showed reticular staining of the lining layer, as in normal synovium, but where hyperplasia of the lining layer was present, staining was less prominent. Several specimens showed blood vessels with thick coats of intense stain extending into the surrounding matrix (Figure 4.5.(c)).

Figure 4.5. Distribution of hyaluronan in diseased synovium. (a) Rheumatoid synovium and (b) osteoarthritic synovium with an inflammatory cell infiltrate showed similar patterns of distribution. (c) Blood vessels in osteoarthritic synovium showing hyaluronan in their walls. (Peroxidase developed with ethylaminocarbazole without counterstain x 10)
4.3.4. Relation of hyaluronan to macrophages and lymphocytes in rheumatoid synovium

Samples of rheumatoid synovium (n=3) were incubated with the binding region probe followed by either Mab EBM11 recognising CD68 as a marker of macrophages or a cocktail of Mabs recognising CD3 as a marker of T lymphocytes and CD22 as a marker of B lymphocytes.

A dense reticular pattern of hyaluronan outlined individual cells in areas of heavy cellular infiltration. This was most prominent in areas of macrophage accumulation with hyaluronan apparently closely applied to the surfaces of individual cells (Figure 4.6(a)). Within lymphoid aggregates, the reticular pattern of stain was patchy and incomplete (Figure 4.6(b)).

Figure 4.6. Relation of hyaluronan to macrophages and lymphocytes in rheumatoid synovium. (a) Macrophages (green) occupy the interstices of a dense network of hyaluronan (orange). (b) Lymphocytes (green) are closely apposed to hyaluronan (orange) in blood vessel walls (arrow) but hyaluronan is scantily distributed through lymphoid aggregates (double arrow). (Indirect fluorescence x 10)
4.3.5. Distribution of cell-associated hyaluronan in cells disaggregated from rheumatoid synovium

Synovial tissues from patients (n=5) with rheumatoid arthritis were enzymatically disaggregated and cytospins of the resultant cell suspension incubated with the binding region probe followed by Mab EBM11.

CD68+ macrophages often showed hyaluronan bound in a thick layer to the cell membrane or sparingly present in the cytoplasm (Figure 4.7.). Many non-macrophage cells showed large amounts of surface and cytoplasmic hyaluronan. In both macrophages and non-macrophages, cytoplasmic hyaluronan, where present, showed a coarse granular pattern. The granules were heterogeneous in size and distributed to the perinuclear region.

Figure 4.7. Distribution of cell-associated hyaluronan in rheumatoid synovial cells. A surface coat of hyaluronan (arrow) and cytoplasmic granules containing hyaluronan (double arrow) were present on certain macrophages (green) and non-macrophages alike. (Indirect fluorescence x 40)
4.3.6. Conclusions of 4.3.

Hyaluronan was distributed throughout synovium but the patterns of distribution were very different in normal and diseased tissues. In normal synovium, hyaluronan was present mainly in the lining layer whereas in rheumatoid tissue it was distributed throughout the bulk of the tissue and away from the surface layer. Hyaluronan was especially prominent in areas of heavy macrophage infiltration but less prominent in areas of lymphocyte accumulation. Osteoarthritic synovium showed a pattern of hyaluronan distribution with features of both normal and rheumatoid. All tissues showed hyaluronan to be present in the outer walls of blood vessels but absent from the intima.

In preparations of cells enzymatically disaggregated from rheumatoid synovium, hyaluronan was present in the cytoplasm and bound to the surface of both macrophage and non-macrophage cells.

4.4. DISTRIBUTION OF CHONDROITIN-4-SULPHATE/DERMATAN SULPHATE (Ch4S/DS) AND CHONDROITIN-6-SULPHATE (Ch6S) IN SYNOVIAL TISSUE

4.4.1. Introduction

The morphological distribution of Ch4S/DS and Ch6S in normal and diseased synovium and rheumatoid nodule was investigated using Mabs whose epitopes were exposed by digestion with chondroitinase ABC. Under these conditions, Mab 286 recognised Ch4S/DS and Mab 383 recognised Ch6S. Results on normal and rheumatoid synovium were compared with those obtained with prior digestion with chondroitinase AC (which digests only DS) in place of chondroitinase ABC.

4.4.2. Distribution of Ch4S/DS and Ch6S in normal synovium

Serial samples of normal synovium (n=6) were incubated with Mab 2B6 or Mab 3B3 with prior digestion by chondroitinase ABC.
In most normal synovia, Ch4S/DS was distributed homogeneously throughout the interstitial matrix of the deep tissue and of the lining layer (Figure 4.8 (a).). However, in one tissue with a densely cellular lining layer, Ch4S/DS staining was reduced in this layer but increased in a band immediately deep to it. Ch4S/DS was absent from blood vessel intima and media but most vessels showed a faint condensation of Ch4S/DS in the adventitia. Ch6S was seen on blood vessels in normal synovium as a dense narrow band underlying endothelium (Figure 4.8(b).) together with, in larger vessels, a diffuse outlining the smooth muscle cells of the tunica media. The interstitial matrix of the lining layer showed staining for Ch6S concentrated in a band at the tissue surface. Where the lining layer was sparsely cellular, Ch6S was conspicuous as a dense narrow band at the tissue margin. With increasing cellularity, Ch6S appeared as a wider, less clearly demarcated band enveloping the synovial lining cells. The deep interstitial matrix of normal synovium showed only a uniformly distributed faint fibrillar blush.

Figure 4.8. Distribution of Ch4S/DS and Ch6S in normal synovium. (a) Ch4S/DS shows homogeneous distribution throughout the tissue. (b) Ch6S is distributed largely to the tissue surface and blood vessel walls. (Peroxidase developed with diaminobenzidine without counterstain x 20)
4.4.3. Distribution of Ch4S/DS and Ch6S in rheumatoid tissues

Serial samples of rheumatoid synovium (n=10) and subcutaneous rheumatoid nodule (n=4) were incubated with Mabs 2B6 and 3B3 after digestion of the tissues with chondroitinase ABC.

The results were as follows:

(i) synovium

In all samples, lining layer Ch4S/DS was consistently reduced compared to deeper layers (Figure 4.9 (a).). Ch4S/DS localised to collagen showed the fibrous elements of the interstitial matrix, both superficial and deep, to be of variable thickness, widely separated and orientated in all directions in contrast to the compact, regular, parallel pattern of normal tissue. Many vessels of a variety of sizes showed Ch4S/DS on the luminal aspect and in some cases throughout the vessel wall. The bulk of the deep interstitial matrix was negative for Ch6S but there were scattered patches of faint staining. Ch6S was also uniformly absent from the lining layer (Figure 4.9.(b).), in marked contrast to normal tissue.

Figure 4.9. Distribution of Ch4S/DS and Ch6S in rheumatoid synovium. (a) Ch4S/DS localised to the fibrous elements of the interstitial matrix and blood vessel walls. (b) Ch6S was present in blood vessel walls but absent from the tissue surface (arrow). (Peroxidase developed with diaminobenzidine without counterstain x 20)
The distribution of Ch6S on blood vessels in rheumatoid synovium was similar to that seen in normal.

(ii) nodule

The fibrous tissue which forms the bulk of the rheumatoid nodule showed moderately dense, coarse fibrillar staining for Ch4S/DS throughout, with the fibres in parallel array (Figure 4.10(a).). Palisading cells surrounding necrotic foci were separated from each other by Ch4S/DS+ fibres which were randomly orientated and disrupted and which largely terminated at the margin of the area of necrosis. A few coarse Ch4S/DS+ fibres traversed the area of necrosis, which was otherwise unstained. Ch6S was distributed solely to blood vessel walls; the telangiectatic vessels surrounding necrotic foci were especially densely stained (Figure 4.10(b).).

Figure 4.10. Distribution of Ch4S/DS and Ch6S in rheumatoid nodule. (a) Ch4S/DS+ matrix fibres are seen terminating in a necrotic focus (arrow). (b) Ch6S is restricted to blood vessel walls. (Peroxidase developed with diaminobenzidine with haematoxylin counterstain x 10)

Control tissues incubated with irrelevant Mabs of the same isotypes as the anti-
chondroitin sulphate Mabs showed no comparable patterns of staining. Control tissues incubated with the anti-chondroitin Mabs without prior treatment with chondroitinase showed no staining.

4.4.4. Distribution of Ch4S/DS and Ch6S in osteoarthritic synovium
Serial samples of osteoarthritic synovium were incubated with Mabs 2B6 and 3B3 after digestion with chondroitinase ABC.

Osteoarthritic tissues generally showed patterns of distribution of Ch4S/DS and Ch6S with features seen in both normal and rheumatoid samples. In osteoarthritic synovium, Ch4S/DS was homogeneously distributed throughout the superficial and deep matrix. Ch4S/DS was absent from blood vessel intima and media but most vessels showed a faint condensation of Ch4S/DS in the adventitia. The bulk of the deep interstitial matrix was negative for Ch6S but there were scattered patches of faint staining. Ch6S was also uniformly absent from the lining layer, in marked contrast to normal tissue. The distribution of Ch6S on blood vessels in osteoarthritic synovium was similar to that seen in normal.

4.4.5. Effects of chondroitinase ABC and chondroitinase AC
Serial sections of normal (n=1) and rheumatoid (n=1) synovium and normal skin (n=1) were incubated with Mabs 2B6 and 3B3 after digestion with either chondroitinase ABC or chondroitinase AC.

All tissues incubated with Mab 2B6 after digestion with chondroitinase ABC showed the described distribution of staining (see 4.4.2., 4.4.3. and 4.5.3.). Prior digestion of normal synovium with chondroitinase AC yielded a similar distribution of matrix staining but with a much reduced intensity. A striking addition was dense staining of individual vascular smooth muscle cells. Rheumatoid synovium digested with chondroitinase AC showed matrix staining of similar distribution and only
slightly reduced intensity. Again, there was moderately dense staining of vascular smooth muscle cells and, in addition, staining of large mononuclear cells in areas of marked matrix disruption. Normal skin digested with chondroitinase AC showed moderate epidermal and very faint dermal matrix staining. Again, there was dense staining of vascular smooth muscle cells and also of the arrector pili muscle. All tissues incubated with Mab 3B3 after digestion with chondroitinase ABC showed the described distribution of staining (see 4.4.2., 4.4.3. and 4.5.3.). In all tissues, prior digestion with chondroitinase AC yielded no staining.

4.4.6. Conclusions of 4.4.

Ch4S/DS and Ch6S, although biochemically closely related, were very differently distributed in normal synovium and each showed changes in distribution in disease (Table 4.4.). In normal synovium, Ch4S/DS was present throughout the tissue matrix whereas Ch6S showed a much more restricted distribution. In rheumatoid synovium, the most striking changes were the presence of Ch4S/DS on the luminal aspect of many blood vessels (where it is normally absent) and the absence of Ch6S in the superficial lining layer (where it is normally present). Osteoarthritic synovium showed features of both normal and rheumatoid. In rheumatoid nodule, Ch6S was confined to blood vessel walls; Ch4S/DS was present throughout the connective tissue matrix surrounding necrotic foci but absent from the centres of necrosis.

Predigestion of tissues with chondroitinase AC in place of chondroitinase ABC, in an attempt to locate DS alone, yielded largely similar patterns of staining, although of greatly reduced intensity. This suggested that DS was present in the interstitial matrix in minor quantities compared to Ch4S. The increased staining of vascular smooth muscle was surprising and may represent irrelevant cross-reactivity.
Table 4.4.

DISTRIBUTION OF CHONDROITIN-4-SULPHATE/DERMATAN SULPHATE AND CHONDROITIN-6-SULPHATE IN NORMAL, RHEUMATOID AND OSTEOARTHRITIC SYNOVUM

<table>
<thead>
<tr>
<th></th>
<th>NORMAL</th>
<th>RHEUMATOID</th>
<th>OSTEOARTHRITIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHONDROITIN-4-SULPHATE/DERMATAN SULPHATE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lining layer matrix</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Deep layers matrix</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Blood vessels - intima/media</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Blood vessels - adventitia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>CHONDROITIN-6-SULPHATE</strong></td>
<td></td>
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</tr>
<tr>
<td>Lining layer matrix</td>
<td>+</td>
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</tr>
<tr>
<td>Deep layers matrix</td>
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<td>-</td>
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<tr>
<td>Blood vessels - intima/media</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blood vessels - adventitia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Key: + = present; - = absent

4.5. GLYCOSAMINOGLYCANS IN NON-SYNOVIAL TISSUES AND ALTERATION IN INFLAMMATORY DISEASE

4.5.1. Introduction

The morphological distributions of hyaluronan, Ch4S/DS and Ch6S were compared in tonsil, skin and skeletal muscle. Alteration in inflammatory disease was investigated.

4.5.2. Distribution of hyaluronan, Ch4S/DS and Ch6S in tonsil

Serial sections of normal tonsil (n=3) were incubated with Mabs 2B6 and 3B3, after digestion with chondroitinase ABC, and with the binding region probe for hyaluronan.
Hyaluronan was present as a delicate, reticular tracery around cells in the T-lymphocyte-dependent areas around follicles. Within follicles, this pattern was patchy. Medium to large blood vessels, but not small vessels or capillaries, showed dense staining for hyaluronan in all layers of their walls except the intima (not shown). Ch6S was confined to blood vessel walls but, in contrast to tissues other than tonsil, the staining was heterogeneous in intensity, often faint and often even absent (Figure 4.11(a)). Ch4S/DS was present on coarse matrix fibres, which were densely stained and distributed in swathes around follicles (Figure 4.11(b)). The fibres were loosely packed, and separated by cords of small mononuclear cells. Most strikingly, there were numerous Ch4S/DS+ blood vessels and serial sections showed many of these to be Ch6S-.

Figure 4.11. Distribution of Ch4S/DS and Ch6S in tonsil. (a) Ch6S. (b) Ch4S/DS. Several Ch4S/DS+/Ch6S- blood vessels can be seen (arrow). (Peroxidase developed with diaminobenzidine without counterstain x 10)
4.5.3. Distribution of hyaluronan, Ch4S/DS and Ch6S in normal skin and changes in inflammatory skin disease

Serial sections of normal forearm skin (n=6) and lesional psoriatic skin (n=5) were incubated with Mabs 2B6 or 3B3, after digestion with chondroitinase ABC, or with the binding region probe for hyaluronan.

The normal samples showed variation in the thickness of the epidermis of from four to six cells. Each glycosaminoglycan showed a characteristic distribution which was similar in all samples (Table 4.5.).

Table 4.5.

MORPHOLOGICAL DISTRIBUTION OF GLYCOSAMINOGLYCANS IN NORMAL SKIN

<table>
<thead>
<tr>
<th>SKIN ELEMENT</th>
<th>HYALURONAN</th>
<th>CHONDROITIN-4-SULPHATE/DERMATAN SULPHATE</th>
<th>CHONDROITIN-6-SULPHATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- stratum corneum</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- viable</td>
<td>+ *</td>
<td>- *</td>
<td>-</td>
</tr>
<tr>
<td>Basement membrane zone</td>
<td>-</td>
<td>NK</td>
<td>+ *</td>
</tr>
<tr>
<td>Dermal matrix</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- papillary</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>- reticular</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dermal organelles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- hair follicles</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- sweat glands</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- sebaceous glands</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- arrector pilli</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>- nerves</td>
<td>+</td>
<td>NK</td>
<td>NK</td>
</tr>
<tr>
<td>- blood vessels</td>
<td>+ (adventitia)</td>
<td>+ (adventitia)*</td>
<td>+(basement membrane)</td>
</tr>
</tbody>
</table>

Key: + = present; - = absent; * = altered in psoriatic skin; NK = not known

Hyaluronan was present throughout the epidermis and dermis but absent from the
basement membrane zone (Figure 4.12.). In the epidermis, it outlined the keratinocytes. In the dermis, the intensity of staining was greater in the papillary dermis compared to the underlying reticular dermis but within each zone the intensity of staining was uniform. Arrector pili showed patchy staining and there was a condensation of stain in the adventitia of blood vessels and around hair follicles. Nerves cut in cross-section showed staining of the perineurium.

Figure 4.12. Distribution of hyaluronan in normal skin. (Peroxidase developed with diaminobenzidine without counterstain x 10)

Ch4S/DS was absent from the epidermis but uniformly present throughout the dermis (Figure 4.13(a)). Blood vessel adventitia was indistinguishable from surrounding matrix by its Ch4S/DS content but the inner vessel walls showed no staining. Hair follicles and arrector pili were negative for Ch4S/DS but there was faint outlining of sweat gland tubules.

Ch6S was absent from both epidermis and dermal interstitial matrix but present as a dense band of stain at the dermo-epidermal junction (basement membrane zone) and outlining hair follicles and sweat gland tubules (Figure 4.13(b)). All
blood vessels showed dense staining of their inner walls and arrector pili was also densely stained.

All psoriatic samples showed the typical alteration in skin architecture with increase in thickness of the epidermis and exaggerated dermal papillae containing blood vessels, which were increased in number. All glycosaminoglycans showed altered distribution compared to normal, but the degree of alteration differed for each glycosaminoglycan. Hyaluronan appeared the least affected, the only difference being loss of the sharp demarcation between epidermis and stratum corneum in hyaluronan content (not shown). Ch4S/DS was present, in contrast to normal, throughout the epidermis with staining of both keratinocytes and matrix of comparable intensity to that present in the dermis (Figure 4.13(c)). Ch6S was lost from the dermo-epidermal junction, which showed only as a barely detectable band of stain, but its distribution to other structures was unchanged (Figure 4.13(d)).
Figure 4.13. Distribution of Ch4S/DS and Ch6S in normal and psoriatic skin. (a) Ch4S/DS in normal skin. (b) Ch6S in normal skin, showing dense staining of the basement membrane zone (arrow). (c) Ch4S/DS in psoriatic skin, showing dense epidermal staining. (d) Ch6S in psoriatic skin, showing loss of staining of the basement membrane zone (arrow). (Peroxidase developed with diaminobenzidine without counterstain x 10)
4.5.4. Distribution of hyaluronan, Ch4S/DS and Ch6S in normal skeletal muscle and changes in inflammatory muscle disease

Serial sections of normal (n=4) and polymyositic (n=5) quadriceps muscle were incubated with Mabs 2B6 or 3B3, after digestion with chondroitinase ABC, or with the binding region probe.

All normal samples showed a regular array of muscle fibres which were relatively homogeneous in size and shape. None of the glycosaminoglycans investigated was detected within the substance of normal (or diseased) muscle fibres (Table 4.6.).

Table 4.6.

<table>
<thead>
<tr>
<th>MUSCLE ELEMENT</th>
<th>HYALURONAN</th>
<th>CHONDROITIN-4-SULPHATE/DERMATAN SULPHATE</th>
<th>CHONDROITIN-6-SULPHATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle fibres</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Basement membrane</td>
<td>-</td>
<td>-</td>
<td>+ *</td>
</tr>
<tr>
<td>Endomysium</td>
<td>+ *</td>
<td>+ *</td>
<td>-</td>
</tr>
<tr>
<td>Perimysium</td>
<td>+ *</td>
<td>+ *</td>
<td>-</td>
</tr>
<tr>
<td>Epimysium</td>
<td>+ *</td>
<td>+ *</td>
<td>-</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: + = present; - = absent; * = altered in polymyositis

The basement membrane was characterised by a fine, continuous band of dense staining for Ch6S closely applied to the sarcolemma (Figure 4.14(a)). Ch6S was absent from the endomysium but this region contained Ch4S/DS (Figure 4.14(b)) and hyaluronan (Figure 4.14(c)). For hyaluronan the staining was fainter and less well-defined than for Ch4S/DS. The perimysium and epimysium of all
samples, normal and diseased, did not contain Ch6S but showed dense homogeneous staining for Ch4S/DS and patchy staining of variable intensity for hyaluronan. All three glycosaminoglycans were present in blood vessels, Ch6S in the inner and Ch4S/DS and hyaluronan in the outer walls.

Figure 4.14. Distribution of hyaluronan, Ch4S/DS and Ch6S in normal skeletal muscle. (a) Ch6S. The basement membranes of adjacent cells are clearly seen as 'tram-lines'. The intervening endomysium (arrow) is unstained. (b) Ch4S/DS. (c) Hyaluronan. (Peroxidase developed with diaminobenzidine without counterstain x 10)
Polymyositc samples showed wide variation in fibre size and shape with a large increase in intervening connective tissue. The most striking difference compared to normal was that the band of Ch6S in the basement membrane/endomysium region varied in thickness and was often incomplete or even absent (Figure 4.15(a).). Hyaluronan (Figure 4.15(b).) and Ch4S/DS (Figure 4.15(c).) content was greatly increased commensurate with the increase in endomysial, perimysial and epimysial connective tissue. Blood vessels, although greatly increased in number, showed unaltered glycosaminoglycan content.

4.5.5. Conclusions of 4.5
As in synovium (see 4.3. and 4.4.), all three glycosaminoglycans showed very different distributions in the tissues examined. In tonsil, the most striking finding was the presence of numerous blood vessels positive for Ch4S/DS but negative for Ch6S. This may reflect the special function in cell traffic of vessels in lymphoid tissue. In skin, hyaluronan was distinguished as the only glycosaminoglycan present in the normal epidermis and the only glycosaminoglycan to be unchanged in distribution in the inflammatory disease, psoriasis. Changes in the distribution of Ch4S/DS and Ch6S in disease were most marked in the epidermis and basement membrane zone, which are normally sites of high metabolic activity and this activity is greatly increased in psoriasis. In skeletal muscle, hyaluronan and Ch4S/DS were similarly distributed and both were increased in amount although unaltered in distribution in inflammatory disease. Ch6S was restricted to the basement membranes of muscle fibres and blood vessels and the most striking change in disease was patchy loss of Ch6S from the basement membrane of certain muscle fibres.
Figure 4.15. Distribution of hyaluronan, Ch4S/DS and Ch6S in polymyositis muscle. (a) Ch6S. (b) Ch4S/DS. (c) Hyaluronan. (Peroxidase developed with diaminobenzidine without counterstain x 10)
4.6. EXPRESSION OF THE EXTRACELLULAR MATRIX RECEPTOR CD44 IN TISSUE AND ALTERATION IN INFLAMMATORY DISEASE

4.6.1. Introduction
Expression of the extracellular matrix receptor CD44 in synovium, skin and muscle and its alteration in inflammatory disease was investigated using immunohistochemical techniques. The distribution of CD44 in relation to hyaluronan, for which it is the principal receptor, was assessed in rheumatoid nodule.

4.6.2. CD44 expression in normal and diseased synovium
Samples of normal (n=3) and rheumatoid (n=3) synovium were incubated with Mab F.10.44.2 which recognises CD44. Also included were cytospins of cells enzymatically disaggregated from rheumatoid synovium (n=2) and a cytospin of normal peripheral blood monocytes.

In normal synovium, the bodies of the lining cells and subsynovial fibroblasts were densely stained (Figure 4.16(a)). The intervening matrix showed many densely stained fibres which appeared to be cellular processes. These were closely packed in the lining layer but more widely separated in the deeper layers were the cells were scattered. Rheumatoid synovium (Figure 4.16(b)) showed staining of almost all cells, including macrophages, fibroblasts, lymphocytes and vascular smooth muscle cells, throughout the depth of the tissue, although the staining was markedly less dense than that seen in normal synovium. In both normal and diseased tissue, endothelial cells were uniformly unstained (Figure 4.16(c)).
Figure 4.16. Distribution of CD44 in normal and diseased synovium. (a) Normal synovium. (b) Rheumatoid synovium. (c) Blood vessel in rheumatoid synovium. (Peroxidase developed with diaminobenzidine with haematoxylin counterstain x 20 ((a) and (b)), x40 ((c)))
Disaggregated rheumatoid synovial cells showed staining of >95% of cells, but the staining was markedly heterogeneous in intensity, with large mononuclear cells being the most intensely stained and giant cells only faintly stained (Figure 4.17(a)). The small number of unstained cells were all mononuclear cells. Normal peripheral blood monocytes showed uniform staining of moderate intensity (Figure 4.17(b)).

Figure 4.17. Cellular distribution of CD44. (a) Disaggregated rheumatoid synovial cells. (b) Normal peripheral blood monocytes. (Peroxidase developed with diaminobenzidine with haematoxylin counterstain x 40)

4.6.3. CD44 in relation to hyaluronan in rheumatoid nodule

Samples of subcutaneous rheumatoid nodule (n=2) were incubated successively with the binding region probe and with Mab F.10.44.2.

The palisading layer of macrophages surrounding necrotic foci and the connective tissue fibroblasts were all densely stained for CD44 (Figure 4.18(a)), the latter cells showing long cytoplasmic processes as noted above (see 4.6.2.). The staining of the palisading macrophages faded away into the necrotic foci where there was no
staining. Hyaluronan was also absent from the necrotic foci but was present as a band within and immediately outside the palisading layer and patchily throughout the surrounding connective tissue. The cytoplasmic processes of the palisading macrophages showed double staining for hyaluronan, as did many CD44+ processes on the connective tissue fibroblasts (Figure 4.18(b)).

Figure 4.18. Distribution of CD44 in relation to hyaluronan in rheumatoid nodule. (a) Edge of a necrotic focus showing palisading macrophages (green) (arrow) with double labelled cytoplasmic processes (yellow) and patches of hyaluronan (red) (double arrow). (b) Blood vessels with surrounding connective tissue. CD44+ fibroblast cell bodies (green) (arrow) are clearly seen with prolonged cytoplasmic extensions double labelled (yellow) (double arrow) for hyaluronan (red). (Indirect fluorescence x 20)
4.6.4. CD44 expression in normal and diseased skin

Samples of normal forearm skin (n=3) and lesional psoriatic skin (n=3) were incubated with Mab F.10.44.2. In normal epidermis (Figure 4.19(a)), the cells of all layers except the stratum corneum showed uniform dense surface staining for CD44. Normal dermis showed dense staining of scattered fibroblasts and, as seen in normal synovium (see 4.6.2.), the intervening matrix showed many densely stained fibres which appeared to be cellular processes. Psoriatic epidermis (Figure 4.19(b)) also showed surface staining of the cells of all layers except the stratum corneum, but the intensity was variable and, in places, CD44 appeared absent. Psoriatic dermis showed fibroblast staining similar to that seen in normal.

Figure 4.19. CD44 in normal and psoriatic skin. (a) Normal skin. (b) Psoriatic skin. (Peroxidase developed with diaminobenzidine without counterstain x 20)

4.6.5. CD44 in normal and diseased skeletal muscle

Samples of normal (n=3) and polymyositis (n=4) skeletal muscle were incubated
In normal muscle, the fibres were unstained, as were the surrounding endomysium, epimysium and perimysium (Figure 4.20 (a).). Vascular smooth muscle cells and scattered macrophages and fibroblasts were uniformly densely stained. Diseased muscle fibres were also unstained, but the greatly-expanded surrounding connective tissue coats showed dense staining of fibroblasts and their numerous closely packed cytoplasmic processes (Figure 4.20 (b).).

Figure 4.20. CD44 in normal and polymyositic muscle. (a) Normal skeletal muscle. (b) Polymyositic muscle. (Peroxidase developed with diaminobenzidine without counterstain x 20)


CD44 was expressed on all the cellular elements of normal and rheumatoid synovium, except the vascular endothelium. In the normal synovial lining layer, the numerous, prolonged cytoplasmic processes of the fibroblastic synovial cells appeared to be CD44+ to their limits. The intensity of the staining in rheumatoid
synovium showed much greater heterogeneity but was generally reduced compared to normal. In rheumatoid nodule, the palisading layer of macrophages expressed CD44, as did the connective tissue fibroblasts, many of which showed double-labelling of their cytoplasmic processes for hyaluronan. In normal and psoriatic skin, staining for CD44 was similarly distributed, with surface staining of the epidermal cells and dense staining of the dermal fibroblasts, including their cytoplasmic processes. The most marked difference observed was in the epidermis where, in normal tissue, the cells were uniformly densely stained while in psoriatic samples, the cells showed staining of markedly heterogeneous intensity. CD44 was present on the fibroblasts and vascular smooth muscle of skeletal muscle tissue but the muscle fibres themselves were negative for CD44. The striking increase in CD44 expression in polymyositis muscle was due entirely to the increase in vascularity and connective tissue.

4.7. OVERALL CONCLUSIONS OF CHAPTER 4

The binding region probe, for which optimal histochemical staining conditions were determined, was shown to be successful in visualising hyaluronan in synovium, both normal and diseased, and in a range of non-synovial tissues. Using specific Mabs, the related glycosaminoglycans, Ch4S and Ch6S, were mapped in the same range of tissues. All three glycosaminoglycans showed different and distinctive distributions in the extracellular space of the tissues examined, with marked alteration in inflammatory disease. These findings demonstrated the marked heterogeneity of tissue matrix and its disruption in inflammatory disease.

CD44, which is the principal receptor for hyaluronan and also binds to chondroitin sulphate, was identified immunohistochemically in synovial and non-synovial tissues. CD44 showed an extensive cellular distribution throughout all tissues examined, with profound alteration in inflammatory disease. The pattern of distribution tended to parallel that of hyaluronan. A striking finding was the
presence of CD44 throughout the prolonged cytoplasmic processes of fibroblasts. Particularly in hyaluronan-rich areas, such as the normal synovial lining.
Rheumatoid arthritis is the commonest inflammatory disease affecting synovium but the cause is unknown. As synovium is the target tissue, it would seem reasonable to look in the synovium for features specific to the disease which might provide a lead in the search for a cause. It is becoming clear that carbohydrates may play important roles in cell-cell and cell-matrix interactions and synovial carbohydrates have generally been little investigated. My hypothesis was that carbohydrates provide a specific impetus to the disease process in rheumatoid arthritis. Reports of abnormally increased expression of terminal GlcNAc on serum immunoglobulin G and of serum antibodies to GlcNAc-containing carbohydrates in rheumatoid arthritis suggested a special role for GlcNAc in this disease and prompted a search for terminal GlcNAc in synovium. Serum levels of the complex carbohydrate, hyaluronan, are elevated in rheumatoid arthritis and an abnormal form of hyaluronan is produced by cultured rheumatoid fibroblasts, suggesting that this GlcNAc-containing glycosaminoglycan also has a special role in the disease. GlcNAc will be discussed first, followed by hyaluronan and the related glycosaminoglycans, Ch4S/DS and Ch6S.

Using histochemical methods and a specific monoclonal antibody, rheumatoid synovium was found in this study to contain patterns of cellular and extracellular terminal GlcNAc which were absent from normal synovium. Cellular GlcNAc was present in both cytoplasmic/membrane and nuclear compartments. The marked differences found were, however, neither tissue-specific nor disease-specific but appeared instead to be associated with the presence of inflammation per se. Nevertheless, morphological localisation of GlcNAc, despite its lack of specificity, showed distributions of interest in elucidating inflammation and its perpetuation. Cytoplasmic/membrane, nuclear and extracellular expression of terminal GlcNAc
will be discussed in turn.

Cytoplasmic/membrane terminal GlcNAc is clearly not a marker of cell lineage. It is absent from normal synovium and is expressed by a variety of cell types, that is, macrophages and non-macrophages, RFD1+ cells and RFD1- cells, at least in rheumatoid synovium. It may instead play a role in the inflammatory process, although not one specific to rheumatoid arthritis. Although several cell types may bear GlcNAc, of those studied only a proportion of each did so. Within each type, the GlcNAc+ cells were otherwise morphologically indistinguishable from their GlcNAc- counterparts. This raises the possibility that any cell may be capable of expressing GlcNAc and that this expression is transient. Certainly, peripheral blood monocytes, which are normally GlcNAc-, may, under the (admittedly non-physiological) stress of culture in vitro, become temporarily GlcNAc+ (Sharif M et al 1989). Expression, in terms of the percentage of cells GlcNAc+, peaked at 24 hours and was undetectable by 72 hours. Cell-surface oligosaccharides are important in recognition, transport and adhesion (see 1.3.2.1.) and exposure of GlcNAc on these oligosaccharides in inflammatory disease may be relevant to such processes.

Alternatively, terminal GlcNAc expressed on the cell membrane may be an epiphenomenon, representing incidental damage ("battle scars") to the glycocalyx by inflammatory mediators. This would certainly explain its expression on multiple cell types in diseased synovium and its absence from normal synovium. Indeed, there is some evidence that the carbohydrate moiety of a glycoprotein may actually protect the protein core from damage, at least by enzymatic attack (Gottschalk A et al 1960). The cell-surface carbohydrates comprising the glycocalyx may therefore function non-specifically to protect important cell-surface proteins from degradation. However, by being themselves degraded in the process, they may also provide a measure of the degree of overall damage and,
ultimately, a signal to turnover. There is evidence to suggest that this mechanism operates in the continuous turnover of serum proteins. Kupffer cells in the liver (in common with other macrophages) carry a receptor for mannose, fucose and GlcNAc (Ashwell G et al. 1982) and ligand binding results in internalisation of the bound glycoprotein with its subsequent reappearance at the cell surface free of ligand. Also, liver damage, presumably associated with impairment of Kupffer cell function, results in a rise in the level of asialoglycoproteins in the circulation (Sawamure T et al. 1984). It would seem reasonable to propose that this mechanism operates more widely and that progressive degradation of the carbohydrate moiety of a glycosylated molecule functions generally as a signal to turnover. Terminal GlcNAc, being rarely found on constitutive oligosaccharides, is clearly suited to subserve such a function.

Finally, there is always the possibility that the membrane terminal GlcNAc identified in these studies is, at least in part, artefactual. The double-labelling experiments looking at the relationship between GlcNAc and markers of macrophages and dendritic cells were performed on cells enzymatically disaggregated from rheumatoid synovium and it is possible that enzyme treatment damaged cell surface molecules and thereby interfered with Mab binding to the membrane. This is, however, unlikely as the enzymes used in these studies, collagenase and DNAase, are specific for collagen and DNA respectively, and should have no effect on cell-surface carbohydrates.

A pan-macrophage marker is an essential tool for histological studies of cell populations and as a preliminary to the double-labelling experiments discussed above, the relative merits as macrophage markers of non-specific esterase, Mab RFD7 and CD68 were investigated. CD68 was found to be the most inclusive marker for use on synovium. It should be noted, however, that even this marker is not wholly specific; it is present (as defined by Mab EBM11 binding) on the epithelium.
of the proximal tubules of the kidney (Kelley PMA et al 1988) and synovial and skin fibroblasts in long-term culture (Worrall JG, unpublished observations). This apparent lack of absolute specificity raises a number of issues. First, it is tempting to assume that, although the best available marker does not capture the "essence of macrophage", there exists an as yet undiscovered marker which does. But what is ultimately important is not what a cell looks like but rather what it does. Markers of function delineate cell populations on physiological grounds and until precise functions can be assigned to the cell markers in present use, they may continue to mislead. Secondly, cell markers are almost exclusively recognised using Mabs and all antibodies may show "cross-reactivity", that is, binding to conformationally similar but unrelated structures (Roitt IM 1984). In such cases, the lack of specificity clearly relates to the Mab rather than the marker. All studies employing antibodies in biological systems are subject to this caveat.

GlcNAc+ cells in rheumatoid synovium occasionally showed the aborising morphology typical of dendritic cells, suggesting a relationship with these cells, which are specialised for antigen presentation. Difficulties in identifying dendritic cells in tissue under light microscopy prompted a reassessment of current methods and this study showed Mab RFD1 positivity in combination with CD68 negativity to be more specific for dendritic cells than RFD1 positivity alone. While this composite marker represents an advance over previous ones, it is unfortunately more unwieldy and compounds the problems, discussed above, inherent in using Mabs to identify cell types. Again, a precisely-characterised functional marker of antigen-presentation is needed.

The presence of intense staining for terminal GlcNAc on the nuclear membrane of the majority of cells throughout rheumatoid synovium is consistent with a high density of nuclear pores in these cells. Terminal GlcNAc is present on the nuclear pore complex (Hanover JA et al 1987) and metabolically active cells contain a
greater density of pores than relatively inert cells (Milligan RA 1986). Nuclear terminal GlcNAc may thus be a non-specific measure of metabolic activity. If this is the case, then the finding of greatly increased staining for terminal GlcNAc on the nuclei of lining cells in normal synovium in comparison to subsynovial cells is of interest. Owing to difficulties in extracting pure populations of synovial lining cells from tissue, few functional studies have been performed and the level of metabolic activity and synthetic capabilities of these cells are largely inferred from cytochemical, histochemical and ultrastructural studies. For example, using cytochemical techniques, high levels of activity of the enzyme uridine diphosphoglucose dehydrogenase, which is involved in the synthesis of components of hyaluronan and other glycosaminoglycans, have been found in normal synovial lining cells together with undetectable activity in subintimal cells (Pitsillides AA et al 1992). This suggests that these cells may synthesise synovial fluid hyaluronan. A high level of nuclear terminal GlcNAc on normal synovial lining cells indirectly supports this suggestion by providing independent evidence that these cells are metabolically very active.

It should be noted that increased pore content of terminal GlcNAc-bearing glycoproteins and increased number of terminal GlcNAc residues per glycoprotein could also explain the findings of this study but whether pore structure is susceptible of such changes is unknown. Terminal GlcNAc may also be present on glycoconjugates of the nuclear membrane unassociated with the nuclear pore complex but there is no evidence that these areas of nuclear membrane undergo modification correlated with cellular activity.

Terminal GlcNAc present in the extracellular compartment of rheumatoid synovium bore no clear distributional relation to the glycoproteins, fibronectin and immunoglobulin G, or to the glycosaminoglycan, hyaluronan, but showed a complex relation to fibrin deposition both on the surface of the tissue and in the deeper
layers. Fibrin deposits appeared to carry terminal GlcNAc on their peripheries (although it was not present within the body of the deposit) and, more interestingly, terminal GlcNAc (of coarse reticular pattern) was often present in a band within the tissue immediately adjacent to the deposits. This suggests that GlcNAc present on molecules in the extracellular space of rheumatoid synovium indicates areas of abnormality and acts as a signal for fibrin formation as an initial attempt at repair. Of relevance here is evidence that the macrophage mannose-fucose-GlcNAc receptor, discussed above, is susceptible of modulation by cytokines (Mokoena T et al /1985), suggesting that the infiltrating macrophages associated with inflammation may be stimulated to recognise this extracellular GlcNAc. A mechanism likely to generate abnormal expression of terminal GlcNAc is damage to susceptible molecules by the action of inflammatory mediators. Such a mechanism is likely to be indiscriminate and to lead to changes in a wide range of extracellular and cell-surface molecules. Given that terminal GlcNAc is not normally expressed on either fibrin or its precursor, fibrinogen, (Doolittle RF 1987) this mechanism may be responsible for the presence of GlcNAc on the surface of fibrin deposits, as well as for its presence on constitutive molecules of the extracellular matrix. It is, of course, also possible that abnormal terminal GlcNAc-bearing forms of normal extracellular molecules are synthesised in inflammation or that terminal GlcNAc, as recognised by Mab GN7, is exclusively confined to an extracellular molecule synthesised only in disease and not under consideration in this study.

Of incidental interest was the finding that, in the small number of samples investigated in this study, deep tissue splitting was constantly associated with fibrin deposition. Tissue splitting has been put forward as the mode of formation of synovial villi (Edwards JCW et al 1983). The role of fibrin is unclear although organisation of fibrinous adhesions has been discounted as the method of formation of tissue bridges (Edwards JCW et al 1983). Rheumatoid synovium is friable and fibrin deposits within the tissue may further disrupt the matrix sufficiently for a
split to form under the mechanical stress of joint movement.

In summary, this morphological study did not support a specific role for terminal GlcNAc in rheumatoid arthritis. Instead, terminal GlcNAc, both cellular and extracellular, was found in a variety of tissues affected by different diseases whose only common factor was the presence of inflammation. Nevertheless, terminal GlcNAc may play a non-specific role in the inflammatory process per se. From its distribution in normal and inflamed tissues, one may speculate that its exposure on both cellular and extracellular carbohydrate-bearing molecules by the indiscriminate action of inflammatory mediators acts as a signal for the need for repair.

The search for extracellular molecules bearing terminal GlcNAc led to a consideration of the ubiquitous matrix constituent, hyaluronan, 50% of which consists of GlcNAc residues. No relationship between hyaluronan and terminal GlcNAc was found but as a preliminary to these studies a method was developed for localising hyaluronan in synovium using the hyaluronan-specific binding region of cartilage proteoglycan monomer. This led to a wider comparative morphological study of hyaluronan and the related glycosaminoglycans, Ch4S/DS and Ch6S, in synovial and non-synovial tissues. These three matrix components were found to be very differently distributed and to show profound alteration in inflammatory disease, prompting speculation on their interactions with the cellular components of the tissue.

In normal synovium, hyaluronan was preferentially distributed to the lining layer. This novel observation may be explained, in theory, in several ways. First, a continuous unidirectional movement of hyaluronan throughout the depth of synovial tissue towards the surface may lead to molecules collecting in the lining layer before being released into the synovial fluid. This proposed mechanism can be
discounted, however, as synovial fluid contains hyaluronan at high concentration and molecules would therefore be required to move against a concentration gradient, apparently without active transport. Secondly, lining cells may have a rate of hyaluronan synthesis no greater than that of subintimal cells but hyaluronan from the synovial fluid may, under the influence of joint movement, become impacted in the superficial layer. Thirdly, synovial lining cells may have a high rate of synthesis of hyaluronan which collects in the lining layer before moving into the synovial fluid. It has not been established whether lining cells synthesise the hyaluronan in synovial fluid but, as discussed above, increased expression of nuclear terminal GlcNAc on synovial lining cells compared to subintimal cells supports lining cells having a general level of metabolic activity higher than that of subintimal cells and increased uridine diphosphoglucose dehydrogenase content of lining cells may indicate increased hyaluronan production.

The localisation of hyaluronan in diseased synovium was markedly different from that in normal synovium but appeared to be related to the degree of inflammation present rather than disease-specific, as similar patterns were found in both rheumatoid and osteoarthritic specimens. Hyaluronan was not preferentially distributed to the lining layer in diseased synovium but was present throughout the depth of the tissue. This finding may have several explanations. First, hyaluronan content in the lining layer may be reduced due to the fact that in inflamed tissues the lining layer becomes populated with macrophages which are thought to produce little or no hyaluronan (Dahl IMS et al 1985). Second, hyaluronan content in the deeper layers may be increased due to the fact that fibroblasts throughout an area of inflammation become activated and increase their synthesis of connective tissue substances and, in particular, hyaluronan. Third, the apparent differences in hyaluronan content may be artefactual; rheumatoid and normal synovia may have similar content per unit weight of tissue but binding sites on the hyaluronan molecules in normal synovium may be inaccessible to the binding region probe. The
matrix of normal synovium appears compact and the hyaluronan present may be extensively bound to other matrix components, such as collagen, with the result that binding sites for the probe are masked. By contrast, rheumatoid synovial matrix appears loose and open, suggesting that the hyaluronan present in the tissue may be less firmly bound to other matrix components.

Tissue hyaluronan may be bound not only to matrix components but also to cells. Macrophages and fibroblasts both within tissue and separated by enzymatic disaggregation showed surface-bound and cytoplasmic hyaluronan. Hyaluronan is synthesised on the outer surface of the cell membrane (Prehm P 1984) and hyaluronan bound to the surface of fibroblasts may represent molecules undergoing active chain lengthening. It is not clear whether macrophages are able to synthesise hyaluronan in large amounts but the surface receptor for hyaluronan, CD44, is found on many cell types, including macrophages, and the surface staining for hyaluronan on macrophages may represent CD44-bound material. Intracellular staining probably represents material taken up by pinocytosis. Macrophages appear not to contain a specific hyaluronidase but the other hyaluronan-degrading enzymes, β-glucuronidase and β-N-acetylhexosaminidase, are present in lysosomes (Roden L et al 1989).

During the development of the method for localising hyaluronan, it was discovered that cetylpyridinium chloride, used alone as a fixative for rheumatoid synovium, led to the formation of precipitates of hyaluronan in all regions of the tissue section. With prior rinsing of the tissue, the precipitates did not form, suggesting that they were produced by the action of the fixative on molecules of hyaluronan which could be removed by rinsing. Hyaluronan is highly water-soluble and rinsing the tissue probably removed free or mobile molecules, leaving behind those molecules bound to tissue components. It has been suggested that the well-recognised phenomenon of early morning stiffness in inflammatory arthritis is due to overnight accumulation
in the joint tissues of hyaluronan which, by attracting water, increases tissue turgor. With the resumption of physical activity, the accumulated hyaluronan is dissipated by joint movement, probably initially to the lymphatic system (Fraser JRE et al 1988). Serum hyaluronan levels have been shown to exhibit diurnal variation (Engstrom-Laurent A et al 1987), with a peak occurring about 2 hours after rising in the morning. It is likely that, under the influence of joint movement, a proportion of the free or mobile hyaluronan molecules identified above enter the bloodstream, via the lymphatic system, and produce the observed morning peak in serum levels. These mobile molecules may also represent a low-molecular weight form of hyaluronan which, merely as a function of smaller molecular size, contains fewer binding sites and is, presumably, therefore less likely to be firmly bound to the matrix. Low-molecular weight hyaluronan has been shown in vitro to enhance cellular processes relevant to the propagation of inflammation (see 1.3.3.4.) and may play such a role in rheumatoid synovium.

Many tissues examined showed hyaluronan present in blood vessel walls, distributed to the media and adventitia. Hyaluronan was not observed in the vascular intima in any tissue, whether normal or inflamed and, interestingly, the hyaluronan receptor, CD44, discussed below, was also absent from this region. These findings suggest that the hyaluronan-CD44 system does not play a major role in cell adhesion to endothelium prior to diapedesis but it may be involved in cell passage through the bulk of the vessel wall. Hyaluronan was especially prominent in the arterial wall where it may be important mechanically, allowing the components of the wall to move relative to one another under the influence of pulsatile blood flow.

Ch4/DS and Ch6S are glycosaminoglycans which differ from hyaluronan only in the position of one hydroxyl group and in the addition of a sulphate group. This study shows that the three molecules, despite their similarities, have restricted and characteristic tissue distributions. Although little is known of the specific
functions of the chondroitin sulphate species, these localisation studies suggest that the individual species subserve very different functions in tissue.

In synovium, the most striking finding was the presence of Ch6S in the lining layer of normal tissue. Its presence at this site was surprising as Ch6S was restricted to basement membranes only in all other tissues examined, both normal and diseased. Normal synovial intimal matrix has been shown to contain Type IV collagen and laminin (Pollock LE et al 1990), which are also major constituents of basement membranes. The presence, therefore, in synovial lining of three recognised basement membrane constituents prompts speculation as to whether the lining contains some form of loose, rudimentary basement membrane-like structure with the functions of structural support and molecular sieving. If such a structure is present, then loss of Ch6S from this region in rheumatoid synovium presumably signifies its disruption.

Ch4S/DS is clearly the major chondroitin sulphate species of the deep interstitium. Ch4S/DS binds collagen (Obrink B et al 1975) and its uniform presence on the matrix fibrous elements showed the ordered array present in normal tissue and also illustrated graphically the disruption of matrix architecture found in rheumatoid synovium. The presence of Ch4S on blood vessels in rheumatoid synovium suggests that these vessels may be high endothelial venules. Ch4S is known to be synthesised by the endothelium of high endothelial venules and is expressed on the luminal surface, where it may play a role in lymphocyte adhesion prior to diapodesis (Aruffo A et al 1990). Vessels with features of high endothelial venules have been described in rheumatoid synovium.

Although one may speculate on the role played by each chondroitin sulphate species in its particular location, there is insufficient knowledge of the biological significance of their minor structural differences to infer why one species rather
than the other should be better suited to a particular role. Nevertheless, the striking zonal distributions of Ch4S/DS and Ch6S in synovium and their differential alteration in disease provide compelling evidence in favour of some sort of functional specialisation by these matrix molecules.

Further evidence for functional specialisation by hyaluronan, Ch4S/DS and Ch6S is provided by their characteristic regional distributions in skin and skeletal muscle and alteration in disease. In skin, hyaluronan showed the most extensive distribution, being present throughout the epidermis and dermis (although not the basement membrane zone). A previous study, using a similar probe for hyaluronan (Tammi R et al 1988), showed hyaluronan confined to the epidermis. This cannot be explained as biochemical assays show that, weight for weight, dermis contains four times the amount of hyaluronan in epidermis (Varma RS et al 1982). In the present study, Ch4S/DS was confined to the dermal interstitium and Ch6S was restricted to basement membranes only. This is consistent with a previous study (Sorrell JM et al 1990). In psoriasis, an inflammatory disease of skin characterised by a marked increased in epidermal cell turnover, the most striking changes were loss of Ch6S from the basement membrane zone and presence of Ch4S/DS in the epidermis. The distribution of hyaluronan appeared unaffected. The basement membrane zone at the dermo-epidermal junction is known to be important in normal functioning of the epidermis; for example, it determines the polarity of the basal cell layer from which the epidermal cells arise by cell division (Gumbiner B 1990). Loss of Ch6S from the basement membrane zone indicates alteration in its structure which may directly affect the mitotic activity of basal cells. The abnormal presence of Ch4S/DS in the epidermal matrix, which is produced by the epidermal cells, indicates that these cells must also have undergone metabolic change.

Skeletal muscle has a very low matrix-cell volume ratio, compared to synovium and
skin. Nevertheless, muscle fibres are intimately associated with the matrix, which forms a thin but complete coat, comprising basement membrane and endomysium, around each fibre. Again, Ch6S was found to be restricted to basement membranes. As in skin, the muscle cell basement membrane plays an important role in the normal functioning of the cells with which it is in contact. In this study, the most notable change in inflammatory disease was that Ch6S was completely lost from the basement membrane of certain cells but preserved on others. In polymyositis, it is known that fibres in various stages of degeneration and regeneration may be found alongside one another (Mastaglia FL et al 1970). It is possible that the degree of disruption of the basement membrane, as marked by Ch6S content, distinguishes irreparably damaged from viable cells. If so, it would seem likely that small cells with a dense coating of Ch6S, and, by implication, an intact basement membrane, are regenerating fibres and those without Ch6S, and presumably with a disrupted basement membrane, are degenerate. A previous study (Bertolotto A et al 1987) looking at the distribution of chondroitin sulphates in diseased muscle found similar alteration in Ch4S but no alteration in Ch6S compared to normal. However, the diseased samples were obtained mainly from patients with various forms of dystrophy with only one sample obtained from a patient with polymyositis.

Matrix effects on cells are mediated by matrix receptors. In this study, interest in the matrix began with hyaluronan; investigation of cell-matrix interactions therefore began with a study of the tissue distribution of the principal receptor for hyaluronan, CD44.

In both normal and inflamed tissues, CD44 was found to mirror the expected distribution of fibroblasts, macrophages, lymphocytes, vascular smooth muscle cells and (in skin) keratinocytes. CD44 was not expressed by skeletal muscle fibres but these fibres, in both normal and diseased tissue, were surrounded by individual coats of hyaluronan. However, it appeared that the muscle fibres were
separated from their hyaluronan coats by basement membranes which did not themselves contain hyaluronan. Muscle fibre-hyaluronan interactions, if indeed they occur, are presumably indirect and utilise pathways separate from that involving CD44. The distribution of CD44 in normal epidermis paralleled that of hyaluronan. Dense staining for CD44 was uniformly present on the surface of cells in all layers except the horny, water-resistant surface layer, the stratum corneum. A surprising finding in psoriatic epidermis was patchy loss of CD44 staining while staining for hyaluronan appeared unaltered from normal. Owing to rapid turnover, psoriatic epidermal cells are less well-differentiated than normal and it is possible that CD44 expression correlates with differentiation of these cells. Inflammatory stimuli may also serve to down-regulate CD44, as discussed below in relation to synovium.

CD44 was absent from the luminal surfaces of endothelial cells in all tissues examined but whether it was present on or absent from the basal surfaces could not be directly determined. The following reasoning, however, suggests that CD44 is absent from this site. One surface of the vascular basement membrane is in contact with the basal surface of endothelial cells and the other is in contact with smooth muscle cells, which express CD44. Given that basement membrane is non-cellular it presumably does not contain CD44. If, therefore, CD44 is present on the endothelial cell base, one would expect the basement membrane to be visualised as a narrow unstained band between the CD44+ endothelium and CD44+ smooth muscle. (In skin stained for hyaluronan, the dermo-epidermal junction is visualised as just such an unstained band between the hyaluronan-positive epidermis and hyaluronan-positive dermis.) Such an area was not seen in the blood vessel wall. Endothelial cells have been shown to interact with hyaluronan, both in vivo (McGary CT et al 1989) and in vitro (West DC et al 1989); presumably these interactions are mediated by receptors other than CD44.
CD44 borne on fibroblasts in several locations demonstrated a dense network of cytoplasmic fibres extending into the surrounding matrix. This was most strikingly illustrated in normal synovium where the lining layer was so densely packed with cytoplasmic extensions of the synovial lining cells that it appeared at low power to be homogeneously stained for CD44. The connective tissue component of inflamed skeletal muscle showed a similar appearance. Fibroblasts in the normal subsynovium appeared morphologically similar to dermal fibroblasts. In both locations, the cell bodies were widely separated but showed prolonged, CD44+ cytoplasmic extensions which frequently appeared to run between cell bodies without interruption. The connective tissue of rheumatoid nodule, which surrounds necrotic foci, also showed this appearance. CD44+ fibroblast cytoplasmic extensions, which often appeared in length to be many times the diameter of the cell body, formed an interconnecting network throughout the tissue and could be seen with double-staining often to be in contact with matrix hyaluronan.

It appears that fibroblasts in several locations penetrate and minutely subdivide the matrix between them with extensions of themselves. These extensions may subserve several functions. They may facilitate direct communication between even widely separated cells. They may allow the cell to interact, both with its cellular neighbours and the surrounding matrix, over a wide area. They may allow the intimate contact with the matrix necessary for co-ordinated turnover and repair. The presence of CD44 throughout these extensions suggests that a major function concerns interaction with matrix and, in particular, its hyaluronan component.

In rheumatoid synovium, CD44 was widely distributed on many cell types but the degree of expression, as judged by the intensity of staining, was markedly heterogeneous with many cells showing decreased expression in comparison with normal. In contrast, a recent study (Haynes BF et al 1991) in which equivalent weights of homogenised tissue were subjected to Western blotting and probed for
CD44 content purported to show that CD44 expression was upregulated in rheumatoid arthritis in comparison to normal. Given, however, that CD44 appears to be purely cell-associated, such studies require control for differences in tissue cellularity. An increase in tissue cellularity may overwhelm a concomitant decrease in individual cellular expression, resulting in an overall increase in tissue total CD44 content. A reduction in surface CD44, possibly mediated by inflammatory cytokines, may allow a fibroblast to release its anchorage on the matrix, "draw in its horns" and take part in activities other than meticulous matrix turnover, such as motility, which may be appropriate to the inflammatory process. It is possible, however, that the reduction in individual cellular expression of CD44 in rheumatoid synovium observed in the present study was only apparent: infiltrating non-fibroblastic cells may constitutively express CD44 at a lower level than the resident fibroblasts and, by diluting out the high CD44-expressing cells, they may give the impression that CD44 is down-regulated when for each individual cell CD44 expression is unchanged. This is unlikely, however, as decreased expression was frequently shown by cells with the morphology of fibroblasts. It is clear that investigation of the CD44 expression of individual cells is required to settle this issue. Certainly, preliminary studies on cultured rheumatoid synovial fibroblasts confirm the reduced expression of CD44 by these cells in comparison to normal (JG Worrall, unpublished observations).

In summary, this morphological study demonstrates the heterogeneity of the extracellular matrix, at least in respect of its glycosaminoglycan content, in synovial and non-synovial tissues but does not support a specific role for hyaluronan, Ch4S/DS or Ch6S in rheumatoid arthritis or other inflammatory disease. It does, however, suggest that these three related glycosaminoglycans subserve very different functions in both normal and diseased tissue and prompts speculation as to the nature of these functions. In inflammatory disease, the matrix is disrupted, again at least in respect of its glycosaminoglycan content, and this
disruption may constitute an early signal, transmitted via cell surface receptors for matrix components, to engage in repair. Glycosaminoglycans, in particular, hyaluronan, may play a protective role by "mopping up" inflammatory mediators, thus limiting damage. In chronic inflammatory disease, such as rheumatoid arthritis, attempts at repair proceed alongside continuing damage, which is presumably stimulated by persistence of the initial insult, and lead to gross derangement of the tissue.

The hypothesis that carbohydrates - at least GlcNAc, hyaluronan, Ch4S/DS and Ch6S - provide the specific driving force to the disease process in rheumatoid arthritis has been refuted. Carbohydrates may play a role in the chronic inflammatory process, once initiated, serving to amplify and perpetuate, but specificity presumably resides in either the distinct pattern of susceptibility factors present or the - as yet unidentified - initial insult.
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APPENDIX I

THE REVISED CRITERIA FOR THE CLASSIFICATION OF RHEUMATOID ARTHRITIS

(Arnett FC et al 1988)

1 Morning stiffness. Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement

2 Arthritis of 3 or more joint areas. At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle and MTP joints

3 Arthritis of hand joints At least 1 area swollen (as defined above) in a wrist, MCP or PIP joint

4 Symmetric arthritis Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs or MTPs is acceptable without absolute symmetry)

5 Rheumatoid nodules Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician

6 Serum rheumatoid factor Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects

7 Radiographic changes Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

Key: PIP = proximal interphalangeal; MCP = metacarpophalangeal; MTP = metatarsophalangeal

For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 - 4 must have been present for at least 6 weeks.
APPENDIX II

PRIMARY STRUCTURE OF TYPICAL PROTEIN-BOUND OLIGOSACCHARIDES

i) N-linked to asparagine

\[ X \quad \text{GlcNAc--Gal--Gal--NeuNAc} \]
\[ \text{Asn--GlcNAc--GlcNAc--Man} \]
\[ \text{Ser/Thr} \]
\[ X \]

ii) O-linked to serine/threonine

\[ X \quad \text{Thr/Ser--GalNAc--Gal--NeuNAc} \]
\[ X \]

Key: Asn = asparagine; Ser = serine; Thr = threonine; X = amino acids; Man = mannose; Gal = galactose; GalNAc = N-acetylgalactosamine; GlcNAc = N-acetylglucosamine; NeuNAc = sialic acid
APPENDIX III

STRUCTURE OF HYALURONAN

D-GLUCURONIC ACID

N-ACETYLD-GLUCOSAMINE

STRUCTURE OF CHONDROITIN-6-SULPHATE

D-GLUCURONATE

N-ACETYLD-GALACTOSAMINE
APPENDIX IV

PUTATIVE SECONDARY STRUCTURE OF CD44

(Goldstein LA et al. 1989)

The boxed region shows sequence homology with the hyaluronan binding region of cartilage core protein.

Key
- potential sites of N-glycosylation
- potential sites of O-glycosylation
* potential sites for chondroitin sulphate linkage
POSTSCRIPT

Parts of this work have been published in the following papers: Sharif M et al 1989; Wilkinson LS et al 1990; Worrall JG et al 1991.

Work initiated in this thesis on the role of hyaluronan in synovial matrix and of CD44 in synovial cell-matrix interactions continues in the Department of Rheumatology Research, supported by two separate project grants from the Arthritis and Rheumatism Council.